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Thermophilic Bacteria and Thermotolerant Yeasts for Ethanol Production

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THERMOPHILIC BACTERIA AND THERMOTOLERANT YEASTS FOR ETHANOL PRODUCTION

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TABLE OF CONTENTS

Page

ABSTRACT xv

RÉSUMÉ xvi

LIST OF TABLES x

LIST OF FIGURES xiii

I. GENERAL INTRODUCTION 1

II. GENERAL ASPECTS OF THERMOPHILY 5

- 1. Definition of Thermophily 5
- 2. Ecological Aspects 8
  - a. Origin and Development 8
  - b. Habitats 10
  - c. Species Diversity 11
- 3. Merits and Limitations of Thermophilic Microorganisms 13
- 4. Uses of Thermophilic and Thermotolerant Microorganisms 17
  - a. Alcohol Production by Thermophilic Bacteria 18
  - b. Production of Thermostable Enzymes 19
  - c. Alcohol Production by Thermotolerant Yeasts 21
  - d. Other Uses of Thermophiles 22

Page

III. EFFECTS OF HIGH TEMPERATURE ON STABILITY OF CELLULAR COMPONENTS 25

1. Components Involved in Protein Synthesis 26
    - a. Deoxyribonucleic Acid (DNA) 26
    - b. Messenger - Ribonucleic Acid (mRNA) 27
    - c. Transfer-Ribonucleic Acid (tRNA) 28
    - d. Enzymes 30
    - e. Ribosomes and Their Constituents 30

2) Proteins 34

3) Membranes and Lipids 40

IV. FERMENTATION ETHANOL 48

1. Alcohols as Fuels 48
2. Energy Balances: Raw Materials and By-Products 51
3. Microbial Features for Ethanol Production 53

V. GENERAL PROPERTIES OF THERMOPHILIC ETHANOL PRODUCING BACTERIA 59

1. Anaerobic Non-Spore-Forming Bacteria 59
    - a. *Thermobacteroides acetoethylicus* 59
    - b. *Thermoanaerobium brockii* 60
    - c. *Thermoanaerobacter ethanolicus* 62

Page

2. Anaerobic Spore-Forming Bacteria 66

a. *Clostridium thermosaccharolyticum* 66

b. *Clostridium thermohydrosulfuricum* 68

c. *Clostridium thermocellum* 73

3. *Bacillus stearothermophilus* 77

4. Other Thermophilic Bacteria 81

VI. THERMOTOLERANT YEASTS 85

1. Growth at High Temperature 85

2. Fermentation at High Temperature 88

a. *Kluyveromyces spp.* 90

b. *Candida spp.* 91

c. *Saccharomyces spp.* 93

VII. ETHANOL PRODUCTION FROM CELLULOSIC BIOMASS 100

1. Characterization of the Cellulase System of *Clostridium thermocellum* 103

a. Cellulosome or Cellulose-Cellulase Complex 107

b. Problems of Measuring Cellulase Activity 109

c. Regulation of Cellulase Activity 110

d. Effect of Physiological Factors on Cellulase Synthesis 112

i. Nutritional Manipulation 112

ii. Temperature and pH 114

Page

iii. Agitation 115

iv. Immobilization 116

e. Genetic Manipulation of Cellulase Production 116

2. Thermophilic Mixed Culture Fermentations 118

a. General Features 118

b. *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum* 120

c. *Clostridium thermocellum* and *Clostridium thermosaccharolyticum* 122

d. *Clostridium thermocellum* and *Thermoanaerobacter ethanolicus* 125

e. General Electric Company Processes 126

f. Other Thermophilic Co-culture Fermentations 128

3. Simultaneous Saccharification Fermentation (SSF) Processes 129

4. Xylan Utilization 132

VIII. CONSEQUENCES OF THERMOPHILIC FERMENTATION 134

1. Microbial Features 134

a. Growth Rates 134

b. Cell Yields 135

c. Ethanol Yields and Production Rates 136

Page

2. Process Features 137

a. Substrate Solubility 138

b. Stress on Hardware 139

c. Removal of Volatiles 139

d. Contamination 140

e. Gas Solubility 141

f. Heating and Cooling 142

IX. APPROACHES TO ENHANCE THERMOPHILIC ETHANOL FERMENTATIONS 148

1. Physiological Aspects 148

a. Thermoadaptation and Nutritional Requirements of Thermophilic Bacteria 148

b. Metabolic Control of Thermophilic Bacteria 152

i. *Thermoanaerobium brockii* 153

ii. *Clostridium thermocellum* 156

iii. *Thermoanaerobacter ethanolicus* 157

c. Effect of High Temperature on Yeast Metabolism and Cellular Composition 160

d. Factors Affecting Yeast Growth and Fermentation at High Temperatures 163

e. Heat Shock Proteins 174

Page

2. Ethanol Tolerance 180
   a. Bacteria 180
   b. Yeasts 188
   c. Yeasts at High Temperature 198
3. Genetic Improvement 203
   a. Yeasts 203
   b. Bacteria 205
4. Fermentation/Process Design 210
   a. Systems of Ethanol Recovery 213
      i. Solvent Extraction 213
      ii. Vacuferm 216
      iii. Gas Stripping Ethanol Purifier 218
      iv. Other Systems of On-Line Extraction 221
      v. Cautionary Note 222

X. GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER STUDY 224
1. Microbial Features 224
   a. Bacteria 224
   b. Yeasts 232
2. Process Features 239

XI. ACKNOWLEDGEMENTS 243

XII. REFERENCES 244

Page

XIII. TABLES 334

XIV. FIGURES 374

XV. APPENDICES 396

LIST OF TABLES

1. Possible Factors Affecting Growth and/or Fermentation at High Temperatures.
2. Classification Systems for Thermophilic Bacteria.
3. Upper Temperature Limits for Growth of Various Microorganisms.
4. Potential Merits of Thermophilic Fermentation.
5. Potential Limitations of Thermophilic Fermentation.
6. Other Uses of Thermophilic Microorganisms.
7. Advantages of Thermostable Enzymes.
8. Possible Physiological Basis of Thermostability of Cellular Components.
9. Liquid Fuel Characteristics.
10. Potential Raw Materials for Fermentation Ethanol.
11. Comparison of Desirable Fermentation Features of Yeasts, *Zymomonas mobilis*, and Thermophilic Bacteria.
12. Mesophilic Bacteria with Ethanol as the Main Fermentation Product.
13. Substrate Utilization and Product Formation of Thermophilic Ethanologens.
14. Properties of Anaerobic Thermophilic Ethanologens.
15. List of Yeast Species with Reported $T_{max} \geq 45°C$.
16. *Kluyveromyces spp.* Capable of Fermentation at Temperatures $\geq 40°C$.
17. *Candida spp.* Capable of Fermentation at Temperatures $\geq 40°C$.
18. *Saccharomyces cerevisiae* Strains Capable of Fermentation at Temperatures $\geq 40°C$.
19. *Saccharomyces spp.* Capable of Fermentation at Temperatures $\geq 40°C$.

20 Other Yeast Species Capable of Fermentation at Temperatures $\geq$40°C.

21. Thermotolerance of Yeast Genera Tested.

22. Ethanol Concentrations of Thermotolerant Strains in Flasks and/or Fermentors.

23. Effect of Temperature on the Kinetic Parameters of *Kluyveromyces marxianus*.

24. Effect of Temperature on the Kinetic Parameters of *Saccharomyces uvarum (carlsbergensis)*.

25. Molasses Fermentation of Various Yeast Strains at High Temperature.

26. Comparison of Mono- and Co-culture Fermentations of Cellulosics by *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*.

27. Comparison of Different Strains of *Clostridium thermosaccharolyticum* with Respect to Product Formation.

28. Effect of Xylose and Cellobiose on Fermentations of Steam Exploded Wood (SEW) Using *Clostridium thermocellum* JW20 Alone and in Co-culture with *Thermoanaerobacter ethanolicus* JW200.

29. Comparison of Specific Ethanol Production Rates (q_p) and Product Yields ($Y_{p/s}$).

30. Effect of High Temperature on Physical and Chemical Properties of Water.

31. Metabolic Regulation in Thermophilic Ethanologens.

32. Effect of H_2 on Glucose Fermentation Products of *Thermoanaerobium brockii*.

33. Relation of Energy Sources to Growth, Fructose Diphosphate and Fermentation Product Yields of *Thermoanaerobium brockii*.

34. Ethanol Formation Rates with Resting Cells of *Thermoanaerobacter ethanolicus* JW200 and Its Mutants JW200 Fe(4) and JW200-L-Large.

35. Effect of Supplementation on Ethanol Yields of *Saccharomyces cerevisiae* Strains 1200 and NP-3.

36. Effect of Linseed Oil and Mixed Fatty Acids Derived from Linseed Oil on Molasses Fermentation by *Saccharomyces cerevisiae* at 30 and 40°C.

37. Possible Detrimental Effects of Ethanol on Microbial Cells.

38. Hypothetical Model to Account for Increased Ethanol Intolerance in *Saccharomyces spp.* at Higher Temperatures as Directed by Membrane Permeability Changes.

39. Potential Advantages of Gas Stripping Ethanol Purifier.

LIST OF FIGURES

1. Potential by-products of cellulosic biomass.
2. Fermentation characteristics of *Clostridium thermocellum* AS39 cultivated at 60°C in cellobiose medium.
3. Aerobic pathway in *Bacillus stearothermophilus*.
4. Hypothetical anaerobic pathway in *Bacillus stearothermophilus*.
5. Fermentation characteristics of *Bacillus stearothermophilus* LLD-15 cultivated at 60°C in 5% (w/v) sucrose tryptone-yeast extract medium.
6. Effect of temperature on D-glucose (4.5% w/v) fermentation by *Candida sp.* HT4.
7. Ethanol production of *Saccharomyces spp.* at 40°C in 20% (w/v) glucose peptone-yeast extract medium.
8. Ethanol production of *Saccharomyces cerevisiae* strains at 40°C and 42°C in 35% (w/v) glucose medium.
9. Major routes of ethanol production from cellulosics.
10. Hypothetical pathway for cellulose degradation by *Clostridium thermocellum*.
11. Effect of stirring and added H_2 on ethanol acetate mole ratio of *Clostridium thermocellum* YS grown on 2% (w/v) cellulose.
12. Cellulose fermentation of 1% (w/v) solka floc in mono-culture by *Clostridium thermocellum* LQR1 and in co-culture with *Clostridium thermohydrosulfuricum* 39E.
13. Growth rates of *Clostridium thermocellum* isolates in the presence of ethanol.

14. Fermentation of solka floc and corn stover by two strains of *Clostridium thermocellum*.

15. Growth rates of *Clostridium thermosaccharolyticum* isolates in the presence of ethanol.

16. Ethanol production by *Clostridium thermosaccharolyticum* strains HG-2 and HG-6 from xylose.

17. Co-culture fermentation of *Clostridium thermocellum* S-7 and *Clostridium thermosaccharolyticum* HG-4 on solka floc (80 g/ℓ) and corn stover (100 g/ℓ).

18. Metabolic pathway of a typical anaerobic thermophilic ethanologenic bacterium.

19. Metabolic control of electron flow by reversible oxidoreductases in *Thermoanaerobium brockii*.

20. Double reciprocal plots of the initial rates of the alcohol dehydrogenase reaction of *Thermoanaerobacter ethanolicus* using ethanol as the varied substrate and with NADP concentrations of 4 mM, 0.5 mM, 0.1 mM, 0.07 mM, and 0.05 mM.

21. Effect of varying starch concentrations on ethanol and acetate formation by *Thermoanaerobacter ethanolicus* wild type strain JW200 and mutant strain JW200 Fe.

22. Growth rates of *Clostridium thermohydrosulfuricum* isolates in the presence of ethanol.

ABSTRACT:

This document considers the use of thermophilic bacteria and thermotolerant yeasts for ethanol production. It reviews general and specific properties of thermophilic ethanol-producing bacteria and assesses their relative merits in ethanol production. The main focus concerns employment of these bacteria in mono- and co- culture fermentations for ethanol production from cellulosics; in particular, the cellulase system of *Clostridium thermocellum* and ways of improving it are examined. This review discusses thermotolerant yeasts and physiological factors influencing their growth and fermentation at high temperatures. Special emphasis is on the multidisciplinary approaches required to develop economical processes for fermentation ethanol production at high temperatures. Relevant topics in this section include: adaptation, nutrition, heat shock, ethanol tolerance, metabolic control, genetic improvement, and fermentation/process design. General aspects of thermophily for both bacteria and yeasts (definitions, ecological aspects, merits and limitations, other industrial uses, thermostability of cellular components, and consequences of thermophilic fermentation) are also discussed.

Key words: *bacteria, cellulose, ethanol, fermentation, thermophilic, thermotolerant, yeast*

RÉSUMÉ:

Le présent document traite de l'utilisation des bactéries thermophiles et des levures thermorésistantes pour la production d'éthanol. Il passe en revue les caractéristiques générales et particulières des bactéries thermophiles productrices d'éthanol et évalue la valeur relative de ces dernières pour la production d'éthanol. L'accent principal porte sur l'utilisation de ces bactéries dans des processus de fermentation en monoculture et en coculture pour la production d'éthanol à partir des produits cellulosiques; on traite en particulier du système cellulase de Clostridium thermocellum et des façons de l'améliorer. Cette étude porte aussi sur les levures thermorésistantes et sur les facteurs physiologiques qui influent sur leur croissance et sur la fermentation à des températures élevées. Parmi les sujets pertinents abordés dans cette partie figurent l'adaptation, la nutrition, le choc thermique, la résistance à l'éthanol, la régulation métabolique, l'amélioration génétique et la conception du processus/fermentation. On discute également des aspects généraux de la thermophilie des bactéries et des levures (définitions, aspects écologiques, avantages et limitations, autres utilisations industrielles, thermostabilité des composantes cellulaires et conséquences de la fermentation thermophile.

Mots clés: bactérie, cellulose, éthanol, fermentation, thermophile,
thermorésistant, levure.

I. GENERAL INTRODUCTION

The use of ethanol as a fuel is not a new concept since it was first suggested almost a century ago. The decline in fermentation ethanol dates back to the 1940's when ethylene was produced on a large scale from petrochemicals. The rapid escalation in prices during the oil crises in 1973 and 1981 has regenerated new interest in fermentation ethanol produced from renewable resources. However, the recent world recession and current low oil prices has resulted in cutbacks to research in this important area. The present oil glut is not a solution to the energy crisis; instead, it appears to have given industrialized nations a false sense of security. In view of increasing world population and decreasing oil reserves, it is only a matter of time before our petroleum supply is reduced and prices begin to rise again.

The technical capability exists globally to produce ethanol from a broad spectrum of resources. The conversion of renewable resources or wastes to ethanol can regenerate our fuel and chemical feedstocks, and in addition, alleviate pollution problems. Canada has the potential over the long term for displacing imported oil with its lignocellulosic reserves to achieve independence from world oil markets. Most processes for ethanol production employ the yeast *Saccharomyces cerevisiae* and its related species. However, since yeasts are currently unable to degrade cellulose, the cellulosics must first be hydrolyzed by acid or enzyme, an extra cost factor.

Thermophilic bacteria have long been considered undesirable by the food and canning industry since they survive routine heat treatments. The ability of thermophilic organisms to grow at elevated temperatures ($T_{max} \geq 50°C$) has been a subject of intensive study for years. Although the physiological basis of life at high temperatures is unknown, it appears to involve a combination of factors that permit organisms to synthesize cellular components (membranes, nucleic acids, and other complex structures) that are functional and thermostable. During the last decade, thermophilic bacteria have been regarded less as nuisances and more as potentially beneficial microorganisms. In fact, their special features are of tremendous potential for the development of many biotechnological processes, including ethanol production. In the 1920's patents were filed to produce fuel ethanol from renewable resources using thermophilic bacteria. Therefore, the idea of exploiting them is not new but just reemphasized 65 years later. The use of some thermophilic bacteria in co-culture with the cellulolytic ethanol producing bacterium *Clostridium thermocellum*, offers great potential for producing ethanol directly from both the hexoses and pentoses in cellulosic biomass. At present the cellulase activity of *C. thermocellum* is limited; however, it is only now starting to receive the attention it deserves. Recent understanding of regulatory steps of this cellulase system and genetic advances in recombinant DNA technology, may soon allow this cellulase to compete effectively with the traditionally used fungal cellulase of *Trichoderma reesei*.

Another fundamental problem limiting thermophilic bacteria is their low ethanol tolerance. As a result of this problem low ethanol and high lactate and acetate yields usually result from substrate concentrations $\geq 2\%$ (w/v). Identification of rate limiting and regulatory steps in the metabolism of thermophilic bacteria has led to significant improvements in ethanol yields by physiological and genetic means. Nevertheless, thermophilic bacteria still need to be further improved before they will be economically suitable for ethanol production. In view of the little time invested in these organisms as compared to yeasts and mesophilic bacteria, the potential exists to enhance ethanol production rates further, such that they may someday compete effectively with yeasts. This will entail a multidisciplinary approach in order to fill in the limiting pieces of knowledge about their physiology, biochemistry, genetics, and about processes for scale up. This is illustrated by the recent conversion of the well known food contaminant, *Bacillus stearothermophilus*, into a suitable organism for ethanol production.

One key objective in any fermentation process is to reduce energy requirements and production costs. Fermentation by thermophilic bacteria or thermotolerant yeasts at higher temperature accomplishes this by minimizing cooling, distillation and mixing costs and by theoretically increasing growth and productivity rates. Thermotolerant yeasts are especially advantageous in tropical countries, where cooling costs are very expensive. In addition, they are paramount for efficient simultaneous saccharification fermentation processes currently being investigated. Although there are no true thermophilic yeasts available, this document will review thermotolerant yeasts capable of growth at ≥45°C and/or fermentation at ≥40°C.

One of the fundamental problems in ethanol fermentation, alcohol intolerance, is accentuated at high temperatures. This results in low ethanol yields and/or productivities at elevated temperatures. There have been however, significant developments in the understanding of the physiological effects of high temperature and ethanol on cell membranes and proteins. Continued research in this area may soon lead to the construction of yeasts and/or bacteria that efficiently produce ethanol at high temperatures. Another means of alleviating alcohol intolerance that will be examined is the development of novel fermentation process designs for continuous ethanol removal.

The advantages and limitations of individual thermophilic bacteria and thermotolerant yeasts that are potentially suitable for ethanol production will be highlighted. Several research groups are currently searching and selecting for novel thermotolerant and thermophilic microorganisms. Other laboratories are attempting to further understand their physiology, biochemistry and genetic systems, while still other scientists are examining process/fermentation designs that are suitable at high temperatures. A major part of this document will emphasize the multidisciplinary approaches needed to develop economical processes for ethanol production at high temperatures.

II. GENERAL ASPECTS OF THERMOPHILY

1. Definition of Thermophily

Temperature is one of the most important environmental variables affecting microbial activity. Microorganisms are generally capable of growing over a wide temperature spectrum, however, for each organism, there exists a temperature range outside of which growth and reproduction will not take place.

Microorganisms have generally been divided into three groups; psychrophiles, mesophiles, and thermophiles, depending on their cardinal temperatures for growth. Cardinal temperatures consist of minimum (T_{min}), optimum (T_{opt}), and maximum temperatures (T_{max}). From a review of the literature, it becomes evident that this classification scheme is not satisfactory. In fact, there is no true definition of a thermophile; what is reasonable for one group of microorganisms may be meaningless for another (eg., bacteria and yeasts).

Many microorganisms which fall into overlapping categories have been classified as thermotolerant mesophiles or as thermophiles, depending on the investigators point of view. In many cases, researchers have reported the highest temperature at which growth was detected. However, in some instances only the temperature at which microorganisms failed to grow were quoted, making it difficult to standardize results. It is also very subjective as to what is defined as relevant growth or as slow growth. Caution is needed in view of findings that the cardinal temperatures can be greatly altered by adaptation (1, 2). It is also known that cardinal temperatures for a given species will vary depending on various environmental and physiological factors (Table 1). Tendler *et al.* (3) in 1967 suggested that "obligate thermophily" may simply be an artifact of laboratory media. Kluyver and Baars (4) implied that many thermophilic cultures were physiological artifacts.

Thermophilic bacteria growing at the low end of the temperature scale (~40°C) have been considered different from those growing in the upper temperature range (>100°C). Consequently, several attempts have been made to subdivide the thermophilic bacteria and some of these are illustrated in Table 2. It can be seen that most schemes define thermophilic bacteria as those that have a T_{max} >50°C. Yet, bacteria that have a T_{max} <50°C are often referred to as thermophilic, when they are used for the production of thermostable enzymes, or are used in the dairy industry (i.e., thermophilic *Lactobacilli*) (13, 15).

Heinen and Heinen (16) has proposed the term "caldoactive" (caldus in latin meaning hot) to describe extremely thermophilic bacteria (T_{max} >70-75°C). Wiegel and Ljungdahl (14) have recently devised the term "barothermophilic" for those bacteria able to grow at temperatures >100°C, when sufficient pressure is applied to prevent water from boiling. Earlier, Stetter (17) devised the term "pyrophilic", after *Pyrodictium*, the first group of bacteria found growing in this temperature range. Aragno (12) labelled all organisms thermophilic whenever optimal growth was in the thermobiotic range (i.e., >45°C). Wiegel (18) recently proposed the terms "temperature tolerant organisms of the thermophilic, mesophilic, or cryophilic growth range", for those microorganisms which could grow over a temperature span significantly more than 40°C.

The use of the term "thermophilic yeast" is even more ill-defined. The term is usually used to describe a yeast which grows at temperatures slightly above the T_{max} of most yeasts. Travassos and Cury (19) defined thermophilic yeasts as those growing optimally at $\geq$37°C. Some papers refer to thermophilic yeasts, where the organism can only grow up to 37°C (20), while yeasts which grow at temperatures exceeding 45°C are referred to as thermotolerant by other workers (21). Cooney and Emerson (22) defined thermophilic fungi or yeasts as those which were capable of growing at temperatures >50°C. Other investigators have referred to yeasts which can grow up to 45°C as "psychrophobic" (23, 24). Recently Watson *et al.* (25) defined a thermophilic yeast as one with a minimum temperature limit of 20°C with no restriction as to the maximal temperature limit for growth. McCracken and Gong (21) defined thermotolerant yeasts as those with a T_{min}, T_{opt} and T_{max} of 20-26, 26-35, 37-45°C, respectively, while mesophilic yeasts had a T_{min} of 5-10°C, T_{opt} of 24-30°, and T_{max} of 35-40°C. However, it becomes evident that these schemes are not appropriate for many yeasts, eg., *Candida macedoniensis* - T_{min} 5°C, T_{max} 45°C, *Saccharomycopsis guttulata* - T_{min} 34°C, T_{max} 42°C (26).

It is the author's view that there is insufficient evidence for the existence of a "thermophilic yeast". This review will deal with thermotolerant yeasts, defined here as those yeasts reported to grow at temperatures $\geq$40°C. The emphasis will be placed on yeasts capable of ethanol production at $\geq$40°C and/or growth at $\geq$45°C. Thermophilic ethanologens (27), thermophilic bacteria capable of producing ethanol as a major product from saccharides (sugars, starch, or cellulosics), will also be examined. Both thermophilic bacteria, defined as those having a $T_{max} \geq 50°C$ and $T_{opt} \geq 45°C$ and extremely thermophilic bacteria with $T_{max} \geq 70°C$ and $T_{opt} \geq 65°C$ will be referred to in the text as thermophilic bacteria. Little attempt will be made to describe thermotolerant bacteria (T_{opt} <45°C).

2. Ecological Aspects

By studying various ecological aspects of thermophilic microorganisms, one hopes to obtain further clues which can help select new potentially useful organisms for ethanol production. The ecology of thermophilic bacteria will be discussed only briefly since it has been extensively reviewed previously by Brock and others (28-35). Other reviews on the subject deal with thermophilic fungi (22) and blue green algae (28, 36, 37).

a. Origin and Development

The existence of microorganisms which grow and require high temperatures has stimulated much speculation and research as to how they evolved and adapted. Unfortunately, the origin and development of thermophilic bacteria is still uncertain (38). Many of the early theories reviewed by Gaugran (39) are intriguing. For instance, Arrhenius (40) thought that thermophiles originated from the planet Venus, and that they travelled to earth by means of the sun's rays. A more logical explanation around that time period, was that thermophiles arose as a result of a single spontaneous mutation in a mesophilic organism (4). However, on the basis of current research, it is evident that many mutations would be required to convert a mesophile to a thermophile (38, 41-45). Other theories about how thermophilic microorganisms were able to grow at high temperatures include (44): low water content, protoplasmic organization, protective coating, composition of the medium, etc. However, there appears to be only one theory to explain thermophily for which substantial evidence has been obtained. According to this theory, thermophilic microorganisms have the capacity of synthesize proteins, membranes,and other complex structures that are able to function at high temperatures (Section III).

Thermophilic bacteria are highly specialized and undoubtedly employ different mechanisms of adaptation and of evolutionary strategies to proliferate. The selective pressure for adaptation to the fluctuating temperatures of solar or self-heating environments may have necessitated the involvement of a resistant stage that permitted survival during times of physical or chemical stress (46). This may explain the predominance of spore-forming thermophiles in these habitats. In the same manner, the general lack of spores in extremely thermophilic bacteria found in geothermal waters, may be due to the smaller selective pressure in these niches, since they have more constant temperature and nutrient conditions.

The diversity of thermophilic microorganisms (i.e., as heterotrophs, aerobes, anaerobes, sulfate reducers, facultatives, etc.) and their similarity to mesophiles in structure and metabolic function, suggests that these two groups may have had a common origin (38, 46-48). It is still uncertain which of the two groups came first, the mesophiles or the thermophiles. It has been suggested that thermophiles first developed and then adapted to grow at lower temperatures; however, since it is generally accepted that water temperatures were lower when procaryotes evolved, mesophiles might have evolved first (30, 35, 39, 47, 49-51).

There has been some suggestion that thermophiles evolved by gradually extending their tolerance limits towards higher temperatures where there was less competition and a better chance for survival (35, 47). In this regard, it may be argued that the term "thermophile" (in Greek, "thermos" - not cold, "phile" - loving), meaning "love of heat" is incorrect (29). It is speculated that the expanded temperature range for growth by some microorganisms was due to the evolutionary assimilation of a second set of critical enzymes which functioned at the higher temperature (14, 52).

b. Habitats

Thermophilic microorganisms can be found in a variety of habitats (33, 46). These include: numerous types of volcanic and geothermal environments such as algal mats where the temperatures often reach 100°C, solar-heated environments such as sediments, soil, or litter, where temperatures can reach 70°C, and biological self-heating habitats such as compost, hay, straw, manure, or coal refuse piles, where temperatures often rise high enough for spontaneous combustion to occcur. The great fluctuations in temperature in both solar and self-heated environments result in a diverse number of species that function at widely differing temperatures (46). In contrast, there is much less microbial diversity in geothermal waters where the temperatures are usually kept much more constant by continuous volcanic activity.

Certain species such as *Clostridium thermohydrosulfuricum* and *Clostridium thermoautotrophicum* are so ubiquitous that they have been isolated from nearly every soil sample from both hot and cold climates (14). However, other microorgansims like *Pyrodictium* and *Sulfolobus spp.* have only been isolated from one location.

Over the last few years, there seems to be a trend for scientists to isolate and characterize new thermophilic organisms from diverse habitats (eg., volcanos, thermal springs, grape musts, sludge) (53-58). There does not appear to be a link between the source of isolation and the potential for fermenting sugars (14). Nevertheless, it is believed that potentially new thermophilic isolates that await discovery may possess novel features advantageous for many industrial fermentations (46, 48, 59). Thermotolerant yeasts have been isolated from various sources (soil, cacti, compost, etc.) and this will be discussed subsequently (Section VI, 1).

c. Species Diversity

Thermophilic bacteria have a limited species composition, as they occur in many different genera, similar to those of mesophilic bacteria (14, 38). Although the diversity of microorganisms which have been cultivated at high temperatures is large, as the temperature is raised, the number of species able to grow decreases markedly (33). From a review of the literature, it also becomes evident that more detailed studies will undoubtedly yield new species. Concerted efforts to isolate and describe new microorganisms have only been initiated recently.

In the past, technological problems such as pure culture growth of thermophilic anaerobic microorganisms, have hampered detailed investigations in the diversity of species. However, new sophisticated methods (60) for culturing anaerobes such as Hungate tubes (61), anaerobic glove boxes (62), more thermostable gelling agents such as silica and Gelrite (63), and temperature gradient incubators (64, 65), have facilitated research investigations.

Although high temperature has a pronounced effect on bacterial species diversity, other environmental factors such as pH, nutrients, ionic strength, etc. also play an important role (31, 33, 66-68). For example, thermophilic bacterial species diversity in thermal acid environments is more limited than in neutral habitats and extreme thermophilic alkalinophiles have not yet been found (13, 46). Brock (33) reported that all thermophilic bacteria fall into two groups; thermoacidophiles - pH optima ~1.5-4, and thermophilic neutrophiles - pH optima ~5.5-8.5. In fact, the upper temperature limit for bacteria decreases with decreasing pH. It appears that the acidity adds an additional stress that makes microbial growth at high temperatures impossible. Brock (31) claimed that at pH 2-3, the upper limit for bacteria was 75-80°C.

Tansey and Brock (34) in 1978 compiled a comprehensive list of well characterized thermophilic procaryotic and eucaryotic microorganisms. An updated list of the many new bacteria since described can be found in Wiegel and Ljungdahl's (14) recent review.

It is clear that the upper temperature limits of microbial life are still undefined (Table 3). Brock (29) postulated earlier that increased hydrostatic pressure may permit growth at even higher temperatures. It has recently been shown that microbial growth could be modified by compressed gases and hydrostatic pressure (69). Under higher pressure, *Bacillus caldolyticus* was adapted to grow at 105°C (70). It was concluded that the boiling point of water was not the upper temperature limit for microbial life, provided the water was kept in the liquid phase under high pressure. Stetter and co-workers (17, 71, 72) recently characterized bacteria (*Pryodictium spp., Methanothermus ferridus*) that were capable of growing at up to 110°C. Baross and co-workers (73, 74) reported growth of "smokers bacteria" (isolated from the depths of the sea) at temperatures up to 250°C. However, Trent *et al.* (75) conducted experiments illustrating that the so called 'smokers bacteria' may have been artifacts produced in the media, or just contaminants. Even though Baross and Deming (76, 77) presented further evidence supporting their original claims, it becomes apparent that this is still a very controversial issue. New organisms isolated from the sea that grow at temperatures up to 350°C may have many potential uses (78). Still, relatively little is known about the entire spectrum of thermophilic microorganisms that may be of potential use for alcohol production.

3. Merits and Limitations of Thermophilic Microorganisms

There are numerous potential merits as well as limitations of thermophilic fermentations. These are briefly summarized in Tables 4 and 5, respectively. Both general and specific advantages and disadvantages of various microorganisms and processes will be discussed in greater detail in appropriate sections (see Section VIII). It should be noted that some of these advantages may result in significant cost savings while others may contribute only marginally to the fermentation process. This will depend on the fermentation process and the microorganism itself.

Thermophilic microorganisms exhibit high metabolic activity at temperatures optimal for growth. The high activity and stability of thermophilic enzymes make them promising biocatalysts for many industrial purposes. The faster growth and fermentation rates are gained at the expense of cell yield. This makes thermophilic microorganisms potentially unsuitable for single-cell protein (SCP) production. However, the energy required for drying the microorganisms for SCP production may be reduced (79, 80). Furthermore, cheaper cooling costs can make SCP production from substrates producing much heat (eg., methanol) more economical (46). On the other hand, the high product to substrate ratio makes thermophilic fermentations potentially very suitable for ethanol production. Unfortunately, the higher ethanol yields and productivities expected with thermophilic microorganisms are often not achieved in reality.

All thermophilic ethanol-producing bacteria form acetic and/or lactic acids as by-products. This reduces the fermentation efficiency and results in lower ethanol yields. Recent experiments however, suggest that organic acids may be easily separated from ethanol during the distillation process (81). One of the major problems in fermentation in general, alcohol intolerance, is accentuated at higher temperatures. With increasing temperature all microorgansims become less and less ethanol-tolerant. Furthermore, growth of all thermophilic wild type bacteria are usually inhibited by as little as 0.5-1% (w/v) ethanol. This is believed to be responsible in part for the low substrate tolerance exhibited by thermophilic bacteria. However, both substrate and ethanol tolerant mutants showing potential promise in this area, have been obtained by various selective schemes (Section IX, 2a).

One of the main advantages of thermophilic bacteria, in addition to their broad pH and temperature range, is that they utilize a much broader range of substrates. The utilization of both cellulose and hemicellulose by some thermophilic anaerobic bacteria makes the potential of ethanol from biomass appear very economical. More basic research is still needed with these potentially attractive microorganisms which were only discovered a few years ago. The limited knowledge of their physiology and biochemistry coupled with the fact that most studies were done on very small scale (Hungate tubes or flasks and not controlled bioreactors), will undoubtedly pose further problems in using them on an industrial scale. In fact, it has been pointed out that the low heat and mass transfer and incomplete mixing in tubes or flasks can cause a significant number of artifacts (82). Furthermore, the stress of the high temperature on biotechnology hardware [eg., dissolved oxygen (D.O.) probes deteriorating, leaching of inhibitory trace elements from steel fermentation vessels, etc.] should not be underestimated (13, 82). There is also very little known about distribution and fitness of the thermophilic microorganisms in new environments such as waste streams or indirectly into various products (14).

For the most part, higher temperatures make many process parameters more favourable for fermentation. Elevated temperatures increase the vapor pressure of volatile compounds. This facilitates ethanol recovery by distillation or by continuous removal from the fermentation broth by novel processes (Section IX, 4a). Although it also enhances removal of potentially inhibitory volatile compounds, it may pose a problem by causing an extensive amount of media evaporation leading to concentration of potentially toxic metabolites.

As a result of decreases in density, surface tension, and viscosity, higher temperatures increase the solubility of most substrates. This decreases the energy requirements needed for mixing and allows higher concentrations of substrates with lower solubilities. The decrease in oxygen solubility at higher temperatures favours anaerobic fermentations but may pose a problem in cultivating aerobic microorganisms.

In general, the use of anaerobic microorganisms eliminates some of the expense required in aerobic processes; namely, aeration, D.O. probes, etc. Furthermore, anaerobic fermentation often results in a lower nutrient requirement, less potential ethanol consumption, and a higher ratio of product formed to substrate metabolized.

Thermophilic fermentations reduce energy costs for maintaining proper temperature controls. Depending on the specific process and microorganism, the fermentor may need to be slightly heated or cooled. The former, due to a higher heat dissipation at elevated temperatures, or the latter due to the high rate of heat production. In this regard, substantial cost savings would result from the use of thermotolerant yeasts in many hot and humid parts of the world where cooling water is a major expense in fermentation processes.

Operation of fermentors at higher temperatures inhibits growth of pathogenic organisms; an advantage especially in the bioconversion of municipal wastes. In addition, the use of extremely thermophilic bacteria as hosts for cloning genes would be safer from the standpoint of hazard (83). From the limited data available, thermophiles may be less inherently stable, as the probability of mutation at high temperatures is also increased (82, 84, 85). Compared to mesophilic bacteria, contamination problems are minimized. However, thermophilic cultures often become contaminated with the spore-formers *C. thermohydrosulfuricum* and *B. stearothermophilus*.

4. Uses of Thermophilic and Thermotolerant Microorganisms

Besides ethanol production, the special features of thermophilic microorganisms give them tremendous potential for other biotechnological processes (Table 6). These include manufacture of chemical feedstocks, enzyme technology, bioconversion of wastes, and leaching processes. These processes will only be briefly mentioned here. The interested reader should consult Wiegel and Ljungdahl's (14) and Sonnleitner's (82) recent extensive reviews on the importance of thermophilic bacteria in biotechnology.

a. Alcohol Production by Thermophilic Bacteria

Besides ethanol (which will be the focus of this review), very few other alcohols are produced by thermophilic bacteria. Significant production of methanol has been reported for *Clostridium thermosulfurogenes* (138). However, it is only formed when this organism is grown on pectin as the carbon and energy source. When grown on 0.5% (w/v) pectin and 0.3% (w/v) yeast extact, *C. thermosulfurogenes* produces 11.3 mM methanol, 1.2 mM isopropanol, 2.2 mM ethanol, 19 mM acetate, 2.8 mM lactate, and CO_2/H_2. *Clostridium thermohydrolyticum* was also reported to produce methanol from pectin (139), however it appears this isolate was renamed *C. thermosulfurogenes* (138) a few years later (Zeikus - personal communication).

Clostridium thermosaccharolyticum is known to produce significant levels of butanol. In continuous culture, under stringent conditions, up to 45 mM butanol can be obtained from starch (14). In addition, a newly isolated thermophilic cellulolytic *Clostridium sp.* produced 20 mM of n-butanol after 150 hours cultivation in a liquid medium containing 1% (w/v) cellulose as the sole carbohdyrate source (140). In 1951, Enebo (141) described *Clostridium thermobutyricum* which fermented carbohydrates at 69°C to butanol, as well as other products.

Among the thermophiles, propanol, isobutanol, n- and iso- pentanol, and hexanol are often produced in trace amounts. This is presumably from amino acids and peptides contained in the yeast extract or peptone components of the media (14). Although, Montenecourt and co-workers (142) have recently initiated a study to isolate natural thermophiles for butanol production, thermophilic fermentation for alcohols other than ethanol does not appear to be too promising at present.

b. Production of Thermostable Enzymes

Enzymes from thermophilic microorganisms that exhibit high activities and stabilities at elevated temperatures, are promising biocatalysts for many industrial applications including hydrolysis of substrates for alcohol production (13, 82, 116, 118, 143). Therefore, they will be briefly discussed here. This is evidenced by the many advantages that these enzymes display over currently used mesophilic ones (Table 7) (13, 46, 82, 118).

All thermophiles are generally considered a prospective source of thermostable enzymes. It should be noted however that not all enzymes from thermophilic organisms will always be very stable. For example, the most thermostable glucose isomerase comes from a mesophile (13). Furthermore, thermolysin, one of the most studied thermostable enzymes (retaining 86% activity after 30 hours at 70°C), is isolated from *B. thermoproteolyticus* (82). This strain has a T_{max} of only 55°C and may be considered a mesophile or just a variant of *B. stearothermophilus* (13, 82). Nevertheless, one could expect a greater probability of finding more thermostable enzymes in thermophiles.

Immobilization of enzymes has gained recent interest in various biotechnological processes; however, only a few enzymes are produced on a large scale (eg., glucose isomerase, penicillin acylase) (117, 144).

Immobilization often increases productivity rate, minimizes contamination, stabilizes enzymes, and reduces the formation of waste (145, 146). The use of thermophilic enzymes may further increase the advantages offered by immobilized enzymes and at least two immobilized thermostable enzymes from *B. stearothermophilus* (ß-galactosidase and cyclodextrin glucotransferase) have been patented (14). Some researchers have already examined the effects of high temperature on immobilized enzymes (112, 147).

Although there are presently few thermostable enzymes produced on a large scale, more and more thermophilic enzymes will probably replace mesophilic ones in the future. One factor contributing to this is that thermophilic enzymes, particularly extracellular ones, can be more easily purified by cloning the genes which code for them into mesophiles and subsequently raising the temperature of the broth (148, 149). Extracellular amylases and proteases from some caldoactive *Bacillus spp.* have been studied (16). In fact, one of the top priorities in enzyme research is to utilize thermophilic microorganisms as hosts for synthesizing thermostable enzymes (150). After characterizing a thermostable α-amylase from *Bacillus licheniformis* (151), Novo Enzymes is now producing and selling this enzyme (which has application in the starch industry) on a large scale under the trade name Termamyl (116). Zeikus has recently applied for patents for several thermostable amylases which have potential application in the starch industry (Zeikus - personal communication). The use of some of these enzymes allows raw starch to be processed directly without the need for gelatinization. Already several patents exist on the use of thermostable enzymes such as proteases (114) (reviewed in 14). Zeikus and Lamed (115) recently patented a process for use of ADH/ketone oxidoreductase from *C. thermosaccharolyticum*. This enzyme can be used for the production of chiral solvents, synthesis of NADPH, coenzyme regeneration, and analytical solvent detection (113). The physiological basis for thermostable enzymes will be discussed in Section III, 2.

c. Alcohol Production by Thermotolerant Yeasts

There are several potential benefits of thermotolerant yeasts for use in production of industrial alcohol including, reductions in cooling and/or distillation costs, faster fermentation rates, etc. However, in the case of the brewing or viticulture industry (fermentation temperature ~15-20°C), optimization of fermentation by higher temperatures is only possible to a limited degree (152). Higher temperature accelerates growth, enzyme activity, and absorption of various substrates by the yeasts (153, 154); but, this is accompanied by the formation of higher alcohols and of other undesirable by-products which exert a negative effect on beer flavour and quality (154-156). Similarly, in wine fermentations, temperatures strongly influence the amount of acetate esters formed which contribute to the wine aroma (157, 158). At too high a temperature "wine sticking" takes place and the fermentation stops (159), however, thermotolerant variant wine yeasts have been isolated (160, 161).

Campbell (162) has recently reported however, that above 25-30°C, undesirable flavor compounds begin to fall again to levels acceptable to the brewer. In fact, in Scotch Whisky distilleries, fermentation temperatures often reach 35°C, without any undesirable off-flavors. Although high temperature fermentations (25-40°C) have not yet resulted in beers that matched the flavor of those produced at 15-20°C, Campbell's (162) preliminary results suggest the acceptability of high temperature fermentations.

The effect of temperature on brewer's yeast strains should not be ignored, as the ability to tolerate higher temperatures would be of practical importance (163, 164). Brewer's yeast strains grown at low temperatures are significantly more temperature sensitive than cells grown at high temperatures (165). Thermotolerant yeasts may also be advantageous for other industrial applications, such as in the leavening process in the baking industry. Modest improvements in thermotolerance of baking strains have been achieved by mutation and selection (166, 167).

d. Other Uses of Thermophiles

Among the other potential chemical feedstocks produced via thermophilic fermentation, acetic acid production and possibly lactic acid may soon become viable processes (121). Acetic acid has for years had many industrial uses as a food additive, spice, fungicide in grain storage, and a variety of chemical processes. Most novel and noteworthy is the recent use of acetic acid as a substitute for NaCl in deicing roads as investigated by the U.S. Dept. of Transportation (168). In fact it is expected that acetic acid production will double in the next few years. Many thermophilic bacteria produce variable amounts of acetic acid as a by-product. In addition, three thermophilic homoacetate producing species are known [*Clostridium thermoaceticum*, *C. thermoautotrophicum*, and *Acetogenium kivui* (121, 169, 170)]. *C. thermoaceticum* isolated from manure in 1942 (171) has received much attention over the last few years and has been extensively reviewed (59, 119-121).

Thermophilic microorganisms can be used in many ways to clean up the environment. Many papers report the use of thermophilic microorganisms for SCP production (86-91). However, the production of SCP via thermophilic processes appears of limited value because of lower cell yields at higher temperatures. SCP production using thermophiles may have some future potential from substrates such as methanol, because cooling costs are a major fermentation expense with methanol(46).

Higher metabolic rates and increased biological purification properties, make thermophilic microorganisms very attractive in large scale operations for waste management processes such as composting and sewage treatment. In addition, anaerobic thermophilic fermentation of waste and sewage sludge for methane production offers great potential. The faster rates of organic digestion and of destruction of pathogens, and the high nitrogenous value of undecomposed residues (i.e., fertilizers) may be even more attractive features than the methane produced as a valuable fuel (46).

The importance of thermophilic microorganisms such as *Sulfolobus acidocaldarius* (109, 110) in leaching processes has received much attention in the last few years, especially in view of the fact that less stringent temperature control is necessary. Thermophilic microorganisms can be potentially used for (see Table 6 for references) 1) solubilization and removal of metal ions from low grade ores to obtain metals; 2) concentration of trace elements from dilute solutions, and hence as potential concentration vehicles for recovery of toxic heavy metals from wastewaters; 3) removal of sulfur compounds from coal and crude oil in order to improve their quality and decrease the threat of acid rain.

III. EFFECTS OF HIGH TEMPERATURE ON STABILITY OF CELLULAR COMPONENTS

The physiological basis for the microorganisms ability to grow at high temperatures remains one of the most intriguing aspects of thermophily. In view of man's wealth of knowledge about the microbial cell, it is rather surprising that the primary damage caused by heat is still unknown. In order for growth and/or fermentation to occur at high temperature, all cell constituents must be stable and functional. Thermophily appears to encompass a range of molecular mechanisms and does not appear to be due to any single factor. The following section will present a brief overview on some of the more important effects of temperature on the function and structure of various cell components (nucleic acids, ribosomes, proteins, lipids, and membranes) summarized in Table 8. Reviews on the effect of temperature on microbial cells have been written (12, 38, 43, 153, 172-176). Other detailed reviews exist on the effects of temperature on constituents of protein synthesis (177-183), proteins (41, 43, 184-190), membranes and lipids (191-195). These studies can also have significant medical implications (eg., cancer therapy, anaesthetic action) (196-198).

The inability of thermophiles, or of microorganisms in general to grow at low temperatures, has been reviewed elsewhere (199-201). It will suffice to state that it is also probably due to a variety of reasons which permit growth at high temperatures. At low non-permissive temperatures, thermophilic enzymes and membranes are non-functional due to structural alterations and fluidity changes.

1. Components Involved in Protein Synthesis

a. Deoxyribonucleic Acid (DNA)

The loss of cell viability on exposure to mild heat can be correlated with the introduction of an enzymatic single strand break in the cell DNA (175). It has also been shown that the ability of a cell to repair this damage depends on its physiological and genetic state. Although evidence is lacking, it has been suggested that perhaps thermophiles have a greater ability to repair their thermal injury than mesophiles (181).

Nucleic acids have a characteristic sharp melting point which rises in proportion to the guanosine and cytosine (GC) content. It has been reported that organisms with higher growth temperatures in the thermo- as well as meso- and psychrophilic range, have a greater GC content (43, 185). The thermal stability of DNA seems to bear no apparent relationship to the ability of microorganisms to grow at elevated temperatures; the base composition and melting temperatures (T_m) of DNA obtained from thermophilic bacteria like *B. stearothermophilus* were similar to those of DNA from mesophilic bacteria like *Escherichia coli*. However, other workers found that the DNA of thermophilic *Bacillus* species showed a consistently higher GC content and higher T_m than the DNA from mesophilic *Bacillus* species; 53% and 92°C for the former versus 45% and 88°C for the latter. Since the melting points for the DNA from mesophilic bacteria already lie much higher than the T_{max} of thermophilic bacteria, the higher T_m's should not really be necessary. Perhaps the DNA undergoes structural changes below the T_m and these changes influence its interactions with proteins (38).

b. Messenger-Ribonucleic Acid (mRNA)

A number of investigations have been made concerning the thermostability of the various RNA species in thermophiles (reviewed in 175, 176, 181). The base composition of mRNA of *B. stearothermophilus* was almost identical with the value obtained for DNA of this organism (175). Since one would also expect the base composition of mesophilic mRNA to be almost identical with those of its parent DNA, it was concluded that the thermal stability of mRNA probably does not play an important role in the ability of thermophiles to grow at high temperatures.

Indirectly, the thermostability of mRNA was studied by determining the endogenous amino acid incorporation in a cell free system of *B. stearothermophilus* i.e., the ability of mRNA to direct protein synthesis in vitro at elevated temperatures (181). It was seen that incorporation at 65°C was greater than at 37°C. In contrast, it was found that endogenous incorporation at 37°C was greater with a similar system from *E. coli*.

Other work supported the theory that thermophily depended on the rapid resynthesis of cellular components (as reviewed in 175, 178). It was demonstrated that the turnover rate for mRNA in *B. stearothermophilus* at 40°C and 60°C was approximately one minute at both temperatures compared with 5-6 minutes for *E. coli* at 40°C (175). These workers suggested that the very rapid turnover of labile mRNA in the thermophile was probably a mechanism for providing a high rate of protein synthesis to facilitate replacement of cell constituents which were denatured when the organism grew at 60°C.

Since the extreme lability of mRNA has been well documented, there is the implication that excessive mRNA degradation could occur resulting in the loss of an essential component of the cellular synthetic processes (202). It appears that information on repair of thermal injury of protein biosynthesis is rather scarce. Although some observations have been made concerning the repair of translation imposed by exposure of cells to high temperatures, no evidence of repair of thermal injury of transcription has been found to allow any meaningful conclusion to be drawn (176, 181).

From the data available, one is tempted to conclude that the DNA and mRNA in general do not possess any unique structure that would serve as an important factor in the thermostability of the various cellular functions, and ultimately to the growth of bacteria at high temperature. However, it should be kept in mind that the nucleic acids of thermophiles may possess unique molecular structures which have not been detected by the analytical procedures currently in use. On the whole, however, the process of transcription displays a relative high thermostability. In contrast, the apparent lower thermostability of translation has been extensively documented for various types of cell.

c. Transfer-Ribonucleic Acid (tRNA)

The base composition of tRNA as well as thermal melting profiles from several *B. stearothermophilus* strains was shown to be very similar to that reported for *E. coli*, (T_m ≅80°C, G+C content ~59%) (175, 181). However the tRNA from the extreme thermophile *T. aquaticus*, had a higher T_m and GC content (86°C, 63.5%).

It was reported that the T_m of tRNA from *Thermus thermophilus* increased with increasing growth temperature (203). Watanabe *et al.* (203) reported finding 5-methyl-2-thiouridine (M^5S^2U) in the sequence G-M^5S^2U-C-G of this extreme thermophile. Furthermore, a linear relationship between the T_m and the mole fraction of M^5S^2U in the tRNA was evident (204, 205). It was concluded that thermostability of the tRNA in this bacterium was controlled in an unknown manner by an increase in the growth temperature. This leads to higher T_m values and a greater content of thiolated nucleotide which is important for the tRNA to function in protein synthesis at high temperatures.

It was reported that the tRNA of *B. stearothermophilus* grown at 70°C had 1.4 times as many methyl groups as the tRNA from cultures grown at 50°C (175, 181). This was due to a three-fold increase in the 2-O-methylribose moieties of the tRNA. Since the thermal melting profiles of the tRNA from cells grown at 50°C or 70°C were similar (indicating a similar degree of secondary and tertiary structure) the significance of these ribose methylations is not yet known.

Changes in the tertiary structure of tRNA have also been considered among possible reasons accounting for the thermostability of initiation of translation (181, 206). An analysis of the stability of the mRNA-ribosome-tRNA complex reconstructed *in vitro* revealed that each of the components, including the state of the tRNA molecules, contributed to the stability. Conformational changes of tRNA molecules beyond the anticodon region have been found to affect the stability of the complex at elevated temperatures (181).

d. Enzymes

Thermophilic tRNA synthetases had similar temperature optima when tested with either mesophilic or thermophilic tRNA (8, 207). This suggested that the high temperature optima were primarily due to the amino-acyl-tRNA synthetase and not to the tRNA itself. Bernstam (181) concluded that the thermolability of amino-acyl-tRNA synthetases was very often responsible for the extreme lability of the initiation of translation at high temperature. Furthermore, it was concluded that changes in the rate of association and the dissociation of subunits of the labile synthetases might explain the detrimental effect of high temperatures on translation.

The aminoacyladenylate enzyme complex from *B. stearothermophilus* is similar in stability to that from *E. coli* (208). However, a DNA-dependent RNA polymerase isolated from *B. stearothermophilus* showed much greater thermostability than that from *E. coli* (209).

e. Ribosomes and Their Components

The physiological basis for the thermostability of ribosomes from thermophiles remains to be elucidated (182). Ribosomal RNA (rRNA) has been shown to be degraded in various heated cells (175). Exposure of mesophilic bacteria to high temperatures caused the degradation of ribosomes, however, only the smaller 30S subunit containing the 16S RNA was selectively damaged. In addition to the demonstrated lower thermostability of the small subunit (210, 211), it has also been shown in reconstruction experiments that the more thermostable large 50S subunit can also impart elevated thermostability to the small one (181, 212).

Nomura *et al.* (213) concluded that the thermostability of ribosomes from *B. stearothermophilus* required properties of both rRNA and proteins that were specific to this thermophile. Ljungdahl and Sherod (185) discussed physiochemical properties of ribosomes and ribosomal proteins.

In order to study the heat stability of ribosomes, comparative studies on the incorporation of lysine and phenylalanine with poly U and poly A as templates, were carried out with crude entracts of *E. coli* and *B. stearothermophilus*, respectively (175). It was seen that the ribosomes of *E. coli* lost 90% of their activity after 5 minutes at 65°C, whereas those of the thermophilic *Bacillus* in fact showed a slight increase in activity (10%) when subjected to the same treatment (175). Others reported similar findings (214, 215).

Studies have also shown that ribosomes from thermophiles like *B. stearothermophilus*, *Thermus aquaticus*, and *Clostridium spp.* undergo thermal denaturation at much higher temperatures than ribosomes from mesophiles like *E. coli* (8, 180, 182, 210). Furthermore, studies have shown that rRNA from thermophiles have a higher GC content and exhibit a higher T_m than rRNA from mesophiles (175). However, there are exceptions to this (178, 184). Stenesh and Holazo (216) suggested that rRNA isolated from thermophiles and mesophiles may have similar nucleotide sequences, but differ in the relative frequence of these sequences. Pace and Campbell (177) studied the thermostability of the ribosomes of 19 different microorganisms with T_{opt} ranging from 20 to 70°C.

With a few exceptions, they found a positive correlation between the thermolability of the ribosomes and the T_{max} of the organisms. They also found that the GC content of the rRNA tended to increase with increasing growth temperatures of most of the organisms studied. However, since the whole ribosome was more heat stable than the isolated rRNA, Pace and Campbell (177) concluded that the interaction between rRNA and proteins must act as a stabilizing factor via a conformational change.

Since the more stable ribosomes of thermophiles were not really reflected in a difference in RNA stability, it was thought that the thermal stability may have reflected a difference in ribosomal proteins (173). Irwin *et al.* (180) in fact concluded that the ribosomal protein was the important factor in determining the ribosome's stability. However, the amino acid composition of the ribosomal proteins, from mesophiles and thermophiles were similar (8). These results, however do not exclude the possibility that differences in primary structures may exist between the ribosomal proteins. Complex conformational changes of ribosomal subunits by heating have been studied (181). Some experimental evidence has supported the hypothesis that the stacking arrangement of ribosomal protein and rRNA in thermophilic ribosomes is different from that found in mesophilic ribosomes (178, 182). The effect of proteins may be to produce protein-RNA complexes of a stable configuration (180). Evidence has been presented that thermophilic ribosomal proteins can stabilize rRNA yielding thermostable ribosomes (183).

Since ribosomes used for studies on thermostability have not been extensively purified (178), thermostability could be a consequence of some stabilizing factor which is bound tightly to the ribosome. Friedman and co-workers (215) observed that the polyamine, putrescine, was more effective in stabilizing ribosomes and rRNA from *B. stearothermophilus* than from *E. coli*. Some extremely thermophilic bacteria like *T. thermophilus* produce unique polyamines, such as thermine, which function both to protect macromolecules from high temperatures and to stabilize the protein synthetic apparatus (46, 217). In this regard, Paulin and Poso (218, 219) recently reported the occurrence of ornithine decarboxylase (ODC) activity in *C. thermohydrosulfuricum*. It appears that the synthesis of ODC is somehow connected to the growth of this thermophile and its activity is strongly regulated *in vitro* by GTP and its analogues. Ribosome thermostability may also be related to the activity of a ribosome-bound nuclease. It has been reported that rRNA from thermophiles was more resistant to attack by ribosomal ribonuclease than rRNA from mesophilic ribosomes (220, 221).

Some evidence exists in favour of the view that alterations of cellular membranes are responsible for impaired translation (reviewed in 181). One putative model for example, assumes that high temperature affects a microtubular structural arrangement that extends from the surface membrane to the ribosomes, ultimately affecting translation (181). Even though the effect of high temperature on the membrane and the inhibition of translation are two events occurring at about the same time in the cell, no direct evidence exists implicating the involvement of membranes in the regulation of translation during stress. It seems that an understanding of the effects of elevated temperature on protein synthesis is far from complete.

2. Proteins

Previous work suggested that thermostable proteins have unique structural properties i.e., were smaller with less ordered structure, more rigid, more hydrophobic *interactions*, less ß structure, etc. (176). However, physiochemical analyses have not shown any consistent unusual structural features in thermophiles. Presently well over 75 proteins from *B. stearothermophilus* alone have been isolated and/or characterized (44). Many, such as α-amylase, have been studied in detail (222-226). A number of studies have demonstrated that the thermostability of enzymes from thermophilic bacteria are much greater than their mesophilic counterparts (189, 227-231). In fact, it now appears evident that proteins from thermophilic microorganisms function in all respects like the corresponding proteins from mesophilic microorganisms, except they are generally more thermostable (38, 185).

Wedler (232) concluded that enzymes from thermophilic bacteria closely resemble their mesophilic counterparts in regard to regulatory properties. Some enzymes are under feedback control only at thermophilic temperatures (41, 232). For example, valine serves as a feedback inhibitor for acetolactate synthase from *T. aquaticus* but only at temperatures >60°C (43, 233). The specific effect of high temperature on regulatory properties of catabolic enzymes in thermophiles has not been well studied, but deserves further investigation (46). Temperature does however play an important role, since it can influence the activity or synthesis of ADH in *B. stearothermophilus* (Section V, 3).

Many experiments on the thermostability of enzymes have been carried out to compare the properties of a given enzyme from a thermophile and quite often an unrelated mesophile. However, even after comparison of enzymes from related *Bacillus spp.* (*e.g.*, LDH from *B. stearothermophilus* and *B. caldotenax*) it is still questionable whether it is possible to elucidate the importance of structural differences simply by comparing the amino acid sequences and the 3-dimensional structure of the enzymes (234, 235). The structural and energetic basis for the higher thermostability and function at high temperatures of thermostable enzymes are still not well understood (190).

Oshima (236) proposed 3 mechanisms for the thermostability of thermophiles. Thermostability is intrinsic and the enzymes of this type are thermostable without any additional stabilizer. Most intracellular enzymes belong in this group. The second type involves extrinsic factors, protective factors such as cations. Many extracellular enzymes belong to this group. The third type is thermally induced as demonstrated by the tRNA from *T. thermophilus* and discussed in a preceeding section (Section III, 1c). Another mechanism where controversial results were reported is that heat denatured cell components are rapidly replaced by *de novo* synthesis (1, 41, 237). Although it is conceivable that at extreme temperatures, there is an increase in denaturation which will result in an increase in resynthesis, the general consensus is that this mechanism is not really responsible for thermophily (38, 43, 238, 239).

The higher thermostability of proteins from thermophilic bacteria is an intrinsic property which depends in part on the composition and/or the sequence of amino acids (44, 240, 241). Ohta (242) reported that hydrophobic and hydrogen bond interactions particularly associated with tyrosine were related to the thermostability of thermolysin. These bonds and interactions can influence the stability of a polypeptide by directing its folding (176). Furthermore, the stability of these bonds increases with temperature up to ~65°C (185). The differences in thermostability are not due to differences in types and relative amounts of stabilizing forces but rather in the cooperative arrangement of these forces (44).

Numerous studies were carried out comparing the hydrophobic amino acid content of thermophilic and mesophilic proteins (41, 230, 243-245). A slight change in the amino acid composition altered the thermostability by additional hydrogen, hydrophobic, or ionic interactions (184, 246). A single amino acid substitution could drastically change the thermostability of a protein, as was demonstrated for tryptophan synthetase and β-galactosidase (176, 246-248).

Increases in basic amino acids (eg., histidine) at key positions in *Clostridia* ferredoxins result in more ionic bonds and greater thermostability (186, 249-252). However, the low basic amino acid content of *C. thermocellum*'s ferredoxin is in disagreement with such a rule (253). Similarly, lactic acid dehydrogenases from thermophilic *Bacillus spp.* contain more polar amino acid residues (eg. arginine, lysine) at the active site, which results in more ionic and hydrogen bonds, and increased thermostability (190, 235). It was recently shown that thermophilic proteins show multiple amino acid replacements that cause an increase in internal hydrophobicity, a decrease in external hydrophobicity and favored helix-stabilizing residues (231, 254). A positive correlation between average hydrophobicity and thermophily for *Bacillus spp.* proteins was also reported.

Although some thermostable proteins have slightly greater hydrophobicity, others do not (185). It is still debatable whether hydrophobic interactions are important in explaining differences in protein thermostability.

Temperature also affects a wide variety of properties of water, some of which very strongly influence the structure and function of cell components (43). Increased thermostability might also result from a decrease in size and secondary structure as demonstrated with α-amylase (41) or extra ionic bonds between subunits (255). It is noteworthy that when a disulfide bond was genetically engineered into T4 lysozyme, this enzyme was much more stable toward thermal inactivation (256). Others have implicated a decrease in cysteine content (257, 258), a greater amount of aggregation (227, 259), or an association with the membrane (176) as the basis for enhancement of enzyme thermostability. With few exceptions, efforts to relate thermostability of proteins to other intrinsic factors as well, have been generally unsuccessful, namely, the amount of hydrogen bonding, increase in apolar interactions, increase in cystine, differences in secondary or quaternary structure of proteins, and the amount of energy required to activate or denature the molecules. It should be emphasized that a high temperature optimum for enzymatic activity does not necessarily imply the presence of a thermostable enzyme; but instead may reflect an increased heat of activation for the substrate or cofactor stabilized enzyme complex (178). These factors have been discussed in previous reviews (43, 176, 188). It is most likely that no single effect alone is responsible for enhanced thermostability, but rather a combination of factors is involved.

Many enzymes from thermophiles are denatured *in vitro* at temperatures at which bacteria normally grow. Consequently, additional stabilization must be afforded by the *in vivo* environment via extrinsic factors; interactions with cellular components such as substrates, cofactors, ions, etc. (176). The role of cations (eg., Mg^{+2}, Ca^{+2}, K^+, NH_4^+) in enhancing thermostability has clearly been demonstrated. Thermolysin, α-amylase, glutamine synthetase, ATPase, and aminopeptidases are examples of enzymes from thermophilic bacteria which are stabilized by metals (260-264).

Glutamine synthetase from *B. stearothermophilus* rapidly loses activity at 65°C when NH_4^+ and glutamate or ATP and glutamate are present (259). However, it has been shown to be very stable at 70°C in the presence of Mg^{+2}, glutamate, and NH_4^+ (262).

Although the glyceraldehyde 3-phosphate dehydrogenase (GPDH) from *B. stearothermophilus* is intrinsically thermostable (from inter-subunit ionic bonds) (188, 265), the GPDH from the facultative thermophile *Bacillus coagulans*, is not, and it is in fact more thermolabile than mesophilic counterparts, as it cannot withstand 37°C (176, 266). The thermolabile form can be converted *in vitro* to a thermostable form capable of activity at 55°C, by increasing the ionic strength by a factor of 1.8, by the addition of $(NH_4)_2SO_4$ or NaCl (52, 265). The amount, specific activity, and physiochemical properties of the enzyme from cells grown at 37 or 55°C were essentially identical (176, 267). The only difference that could be related to the thermostability conferred by increasing the ionic strength was an increase in the number of glutamate residues (267). Since GPDH is thermostable *in vitro* at high ionic strength, it was proposed, that proteins from facultative thermophiles, in general, may be stabilized *in vivo* by a highly charged macromolecular environment (186, 265, 267). This enzyme represents an ideal system for evaluating changes in conformation and activity for uncovering new information on thermophilic mechanisms.

The role of cations in enhancing thermostability of enzymes is best exemplified by the role of calcium in protecting thermolysin in *B. stearothermophilus* against high temperatures (261). Voordouw *et al.* (268) concluded that the total kinetic thermostability of thermolysin is due to a single calcium binding site, where the affinity constant for binding of the ion is high at 80°C. Dahlquist *et al.* (269) concluded that the major role of calcium in thermostabilization of thermolysin involves protection from a cooperative conformational modification of the region near one or more sites. Yutani and co-workers (248) concluded that the difference in thermostability between thermophilic and mesophilic α-amylases was not directly correlated with the binding of calcium ions. Such a system of stabilization has not been found to be the case for intracellular enzymes and it is unknown whether or not it is a general mechanism for extracellular enzymes.

The uptake rate and the final amount of calcium in *B. stearothermophilus* cells increases with temperature (270). In fact, studies have shown that calcium is not just accumulated as a consequence of, but is required for the thermophilic growth of *B. stearothermophilus* (271, 272). The role of calcium is still unclear at present. Calcium may act by increasing the stability of the cell membrane (273). Alternatively, it might protect enzymes against the action of heat by reversible reactions by forming links within the protein molecules, or by blocking hydrophilic groups of these proteins and thereby decreasing their hydration (271, 272).

In summary, it appears that both intrinsic differences in macromolecular structure and the presence of thermostabilizing factors contribute to the thermostability of proteins from thermophiles.

3. Membranes and Lipids

There is strong evidence in the literature for the existence of a thermostable cytoplasmic membrane in thermophilic microorganisms. The cytoplasmic membrane consists of a phospholipid bilayer containing peripheral proteins which are loosely bound to the membrane and integral proteins which are embedded in the membrane (175). The melting point of a membrane is influenced by the composition of phospholipids, the class of neutral lipids, the length of the fatty acid chain, the degree of its saturation, and the charge and size of the fatty acid polar head (38). The structure of bacterial (274) and yeast lipids (275) will not be covered here as they have been previously reviewed.

As early as 1924, Heilbrunn (276) reported that thermophilic microorganisms had lipids with higher melting points than non-thermophilic organisms. In fact, it was suggested over a half a century ago that the melting temperature of cellular lipids might determine the upper temperature limit for growth (267, 277). Brock (29) postulated that an increase in the percentage of saturated and branched chain fatty acids with an increase in temperature, might enhance membrane stability.

Many investigators have reported that the fatty acid composition of cellular lipids can respond to changes in environmental temperature. Raising the temperature produces a shift in fatty acids which generally have higher melting points and this enhances the thermostability of the bacterial membrane (194, 278-281). This is often accompanied by an increase in saturated fatty acids at the higher temperature, however, there are exceptions to this (282, 283). Straight chain fatty acids have as a class, higher melting points, and these increase with the length of the carbon chain (44). However, there is no simple correlation between the chain length of a fatty acid and its T_m (175). The molecular geometry of lipids is also of great importance for thermostability (284). As a general rule, isobranched fatty acids have higher melting temperatures than anti-isobranched ones, which in turn, have higher melting temperatures than fatty acids containing cis-double bonds (193).

Unusual lipids are found in the thermo-acidophiles such as *Thermoplasma acidophilum* (43). The cell membrane of this organism has low levels of fatty-acyl residues and is constructed in a manner that can withstand both high temperatures and acidic pH. Growth at high temperatures is related to long isopropanol chains, while growth at acidic pH is related to the presence of lipids with ether linkages and the marked reduction in chargeable groups of membrane proteins (210, 285). The exceptionally high carbohydrate content of the lipids of extreme thermophiles such as *Thermus spp.*, may also be of significance in relation to the molecular basis of thermophily (286).

Data indicates that thermophiles generally contain a higher content of saturated and branched chain fatty acids than mesophiles (279, 287-290). It is also now firmly established that yeasts produce more saturated fatty acids and/or regulate the chain length of fatty acids with an increase in growth temperature (24, 291-299). However, it has been reported that the lipids of some yeasts are not altered in fatty acid composition, regardless of the temperature at which they grow (300-303). It appears that the lipids of organisms which have evolved to grow at lower temperatures demonstrate fatty acids which have relatively low melting points. The general nature of these differences in fatty acid composition between psychrophiles, mesophiles, and thermophiles, presumably represents long-term adaptations to different thermal environments (193).

Temperature is not the only factor that can change fatty acid composition. The change in lipid composition of microorganisms may also be an indirect effect resulting from a change in other chemical and physical properties of the environment. The fatty acid distribution pattern is also influenced by the physiological age of the culture (289, 304-310). Furthermore, increased oxygen tension has been reported to elevate unsaturated fatty acids of various organisms (24, 295, 304, 306, 308, 311). Since higher temperatures also decrease oxygen solubility, it is possible that this is partly responsible for the greater number of saturated fatty acids observed at elevated temperatures. However, after examination of cells grown at high temperature in a chemostat with controlled dissolved oxygen, it appears that the changes in lipid composition are mostly related to the higher temperature (312, 313). The fatty acid composition of membrane lipids in microorganisms can also vary with the composition of the growth medium (314-316). For example, the fatty acid composition of the membrane lipids of *Acholeplasma laidlawii* can be changed in a controllable manner by supplementing fatty acids in the growth medium (317). However, in a medium deficient in lipids, this bacterium lacks the ability to alter its fatty acid composition. These studies re-emphasize that it is essential to carefully take into account the physiological conditions (nutrients, oxygen level, age of culture, etc.) when an attempt is made to define the cardinal growth temperatures of microorganisms.

It has not yet been established by which mechanism microorganisms regulate the synthesis of their fatty acids. Some results gave no evidence that the increased synthesis of unsaturated fatty acids was in response to an "inappropriate membrane fluidity" (318). The alteration in fatty acid content may not reflect a difference in heat resistance but might be ascribed to the effects of increased temperature on the biosynthetic capacity of desaturating enzymes (316, 319, 320). The higher proportion of unsaturated fatty acids at the lower temperature has been thought to be due to an adaptively altered specificity of the cellular acyltransferases or a change in the relative availability of different substrates for esterification (321).

The manipulation in fatty-acyl composition with temperature [termed homeoviscous adaptation (322)] allows microorganisms to produce membranes with the same constant fluidity at their growth temperature. The existence of such an adaptation mechanism could provide at least two potentially important evolutionary advantages to microbial systems (193). In terms of short-term adaptation, it would permit cellular growth to occur over a wider temperature range. In addition, it would facilitate the evolutionary development of microorganisms capable of normal membrane function in either extremely hot or cold niches. One temperature-sensitive mutant of *B. stearothermophilus*, which was unable to grow above 58°C, was not able to regulate its fatty acid composition at elevated temperatures like the parent strain (323). This provides further evidence that alterations in fatty acid composition at elevated growth temperatures may be an essential feature of thermophily i.e., it may explain the abrupt halt of growth above the T_{max}.

Studies have revealed unusual techoic acid-like polymers in the cell walls of *B. stearothermophilus* and *B. coagulans* grown at 55°C (324-326). It was reported that at higher temperatures, the synthesis of cell wall peptidoglycan increased while techoic acids decreased in themophilic *Bacillus spp.* (266, 325). In addition, Novitsky *et al.* (266) reported that cell walls of *B. coagulans* bound more magnesium at higher temperatures. It was suggested that alterations in cell wall components might influence the susceptibility of an organism to heat i.e., the cell wall may act as an insulator to protect the membrane from thermal injury. However, from the limited available data, it appears unlikely that the cell wall plays a significant role in protecting the cell from thermal injury (175).

An important feature of the lipid bilayer in procaryotes is its ability to undergo a reversible phase transition from a thermotropic gel to a liquid-crystalline state (320, 327-329). The accuracy of the temperature of this phase transition which has been determined using spin labelling techniques and differential thermal analysis has been under debate (191, 192, 329). Since the gel to liquid-crystalline phase is essentially a hydrocarbon chain melting phenomenon, the temperature of this phase change is dependent on the fatty acid composition of the lipid bilayer. Therefore, an increase in the relative proportions of saturated fatty acids at higher temperature, would raise the temperature of this phase transition (193). Since the fatty-acyl composition can change considerably before membrane functions are affected, there is no obvious necessity for fatty acid composition to alter with temperature (192). Furthermore, since the cytoplasmic membrane appears to be stable above the growth temperature, this suggests that the decline in growth rate (above the optimum) is not due to membrane instability (176). However, the physical state of the membrane lipids can be crucial in determining the temperature range of microorganisms in which growth can occur, and the rate of growth within that temperature range (193).

Esser and Souza (330, 331) had proposed that the T_{min} and T_{max} values of *B. stearothermophilus* were regulated by the phase separation of the lipid bilayer. They thought that the temperature where all the lipids were in the liquid and solid state correlated with T_{max} and T_{min}, respectively. However, it has been reported that various microorganisms were able to grow well above the upper boundary of this gel to liquid-crystalline transition state (44, 193, 317). Nevertheless, the physical state of the membrane lipids could influence the T_{max} in a number of ways (43). The general view is that at least a portion of the membrane needs to be in the liquid-crystalline state and none has to be in the gel state, for growth to occur (44, 176, 328). This suggests that the change in this transition may only be important for setting the T_{min} and does not determine the upper temperature limit of growth (43, 193). The necessity for lipids to be in this liquid-crystalline state may be related to the low membrane permeability when it is in the more rigid gel state (237, 332). This could result in the restricted movement and altered availability of the active site of membrane bound enzymes as was shown for the ATPase in *E. coli* (333). Alternatively, the physical state of the membrane lipids could determine the T_{max} indirectly by influencing the conformation of the membrane proteins (193, 334).

Esser and Souza's (330, 331) results suggested that a high percentage of membrane proteins were interacting with each other rather than the lipids. It was concluded that membrane thermostability was also associated by the thermostability of the peripheral and integral proteins. Halvorson and co-workers (335) showed that membrane proteins were randomly dispersed above the temperature of the upper boundary of the phase transition and formed network like patterns at lower temperatures. Below the temperature of the lower boundary of this phase transition, no proteins were observed (335). These results indicated a temperature-dependent interaction between membrane proteins and lipids. The importance of proteins in thermostability is also evident from the increased protein to lipid ratio in *B. stearothermophilus* when it was cultivated at higher temperatures (336, 337). This may simply reflect changes in metabolic pathways as discussed previously in the *B. stearothermophilus* section. In addition to the effects on permeability, the physical state of the lipids may be important for the function of certain integral proteins (334, 338).

It has been proposed that membrane lipids may provide a protective thermal insulation to other cellular components as was clearly demonstrated in the case of alkaline phosphatase (176, 337). Spheroplasts of *B. stearothermophilus* in contrast to spheroplasts from mesophilic bacteria, were exceptionally stable to osmotic lysis and high temperatures (314). Thermal stability of *B. stearothermophilus* spheroplasts was enhanced as the culture temperature was increased and thermostability was maintained by divalent cations (337). This was correlated with an increase in both the membrane content of the cell and the protein content of the membrane. However, there was a decrease in the lipid content of the membrane. It was recently shown that heat treatment resulted in the disorganization and the release of part of the outer membrane in *E. coli*, and as a consequence, resulted in the partial disruption of its permeability-barrier function (339).

In summary, it appears that in order for microorganisms to grow or ferment at elevated temperatures, all of the cellular components must be stable and functional under these conditions. It appears that thermostability of the various cellular constituents involves many complex molecular mechanisms (summarized in Table 8).

IV. FERMENTATION ETHANOL

1. Alcohols as Fuels

The production of alcohol by fermentation is an art which has been practiced for thousands of years. Ethanol, the alcohol consumed in beers, wines, etc. can also be used as a solvent, antiseptic, fuel, etc. (340). Furthermore it can be used as a basic chemical for synthesizing many other products (ethylene, acetaldehyde, acetic acid, acrylic acid, etc.) (341).

The use of ethanol as a motor fuel is not a new concept since as early as 1890, the value of ethanol as a substitute for gasoline was indicated (342). In fact, Monier-Williams (343) book on power alcohol published in 1921, reviewed significant developments in the field for that time period. Alcohol has been used as an additive to gasoline and sold in the U.S. as gasohol; 10% blend by volume of anhydrous ethanol with 90% by volume of gasoline (344). More recently, it has been referred to as an octane enhancer; i.e., it improves the antiknock quality of the fuel and reduces the carbon monoxide content of exhaust gases (344, 345). This is important in the U.S. where the use of lead in gas is being phased out for environmental reasons (346).

Since 1933, ethanol has been employed as a fuel in Brazil, where there are presently more than 400 fuel alcohol plants (347, 348). Most of Brazil's cars are running on at least 17% alcohol blends, while many run on alcohol alone (341). One of the goals of Brazil is to produce >10 billion liters of industrial ethanol by 1985/1986 (349). Currently, Brazil is producing up to

2.5 billion gallons of ethanol per year (350). A number of other countries (Phillipines, Costa Rica, Argentina, South Africa, etc.) are presently pioneering the large scale use of alcohol for fuel (341, 346, 348).

In the early 1900's ethanol was produced mainly via fermentation. The decline of fermentation ethanol dates back to the 1940's when ethylene was produced on a large scale from petrochemicals (341). Ethanol was produced mainly by the synthetic route - the catalytic hydrolysis of ethylene. The recent and rapid escalation in prices during the oil crises in 1973 and 1981 have generated new interest in studies of fermentation ethanol, especially from cellulosics (Section VII). Although, the fermentation industry was predicted to grow significantly in 1981, the present day low oil prices and economic recession, have delayed progress on many projects (351, 352). However, as concluded recently by the Canadian International Development Research Centre, the oil glut is not a solution to the energy crisis (353)! It is only a matter of time before our non-renewable petroleum supply is reduced and prices rise again. In fact, understanding non-renewable resource supply is a complex matter (354). Canada has the potential over the long term, for displacing imported oil with its lignocellulosic reserves (355-357); however, in order to trim their deficit, the Federal government is currently reducing research and development efforts in renewable energy (358). Japan is vigorously pursuing research in this area. Backed with ~100 million dollars of government support, the Research Association for Petroleum Alternatives Development (RAPAD), composed of ~20 companies, is presently developing technology in alcohol from biomass (Oda - personal communication).

Among the lower alcohols, the two prime candidates for replacing petroleum fuels are methanol and ethanol (341, 342). Methanol can be produced from cellulosic biomass by gasification. However, at present all commercial methanol is made from natural gas (mixture of CO_2 and H_2 under pressure) (341). It has been argued that methanol production from wood brings about more significant energy savings than ethanol production (359). Methanol is relatively rare among the major fermentation products of microorganisms. It occurs primarily as a metabolite during methane utilization as well as during anaerobic degradation of biopolymers containing methoxy groups (360).

Butanol and butanediol are the only other described major alcohols produced in high yields by fermentation other than ethanol. The mesophilic acetone-butanol producer, *C. acetobutylicum*, has been studied for a long time; however, ever since the first patent by Weitzman in 1915, no significant improvement in butanol tolerance (≤2% w/v) has been made (361). Similarly, some organisms are capable of producing butanediol as a major end product (eg., *Bacillus, Klebsiella, Aerobacter, Serratia*) (362).

To be competitive with conventional fuels, alternate fuels should have the following properties (342, 363): 1) fuels must be capable of being stored for long time periods, 2) handling should be as comparatively safe as conventional fuels, 3) storage, transportation, and distribution of fuels used should be economical, and 4) fuels should not impose major engineering changes to processes. There is considerable controversy about the effects of methanol in automobile engines; it appears however, that with no readjustment of the carburetor, there is a significant reduction in gas mileage (342).

Furthermore, with methanol gasoline blends, studies have shown problems with starting, hesitation on warmup, and clogging of filters. Propanol and butanol have been used as fuels on a limited basis, the former has been used as an antifungicide in jet fuel, while the latter has had more direct use in automobile fuel (342).

Table 9 compares some characteristics of ethanol, methanol, and butanol with those of gasoline and diesel oil. Petroleum fuels are mixtures of many different chemicals with wide boiling ranges, and thus are advantageous in cold climates (341) . The lower boiling components are volatile enough to allow engine starting at low temperatures. Unmodified engines powered by pure alcohol will not start below 10-15°C. However, it has been debated whether 20% ethanol (v/v) can be mixed with gasoline and used in unmodified engines without difficulties in cold weather, especially in Canada (341, 363). The presence of oxygen in the alcohols gives them compatibility and affinity for drawing water from the air; a characteristic that can cause problems. However, the lower heating values of alcohols are advantageous. The alcohols, especially methanol, also have cleaner burning properties and produce less atmospheric pollution than hydrocarbon fuels (342).

2. Energy Balances: Raw Materials and By-Products

The technology to produce ethanol from a broad spectrum of resources has been around for some time. Virtually any carbohydrate containing material (saccharides) can be ultimately fermented to ethanol; sugars, starches, and cellulosics. Table 10 illustrates some of the more common raw materials that have been proposed for use in ethanol production (341, 346, 364). Few of these raw materials have been used in commercial processes since they do not meet the necessary requirements; yearly continuous supply of low cost raw materials of reasonable uniform quality (346, 365). At present the most promising raw materials are sugar cane and grain (341, 346, 366, 367). However, significant breakthroughs in the future may make ethanol from cellulosics economical (Section VII).

Various articles exist summarizing current views of process economics and factors that determine the cost of fermentation ethanol (364, 368-370). Raw material costs make up about 60-70% of the final ethanol cost (348, 371, 372). This suggests that major breakthoughs will depend on the use of less expensive feedstocks such as cellulosics. Nevertheless, over half of the process energy requirement is steam for distillation (373). It is widely accepted that one of the main goals of process development in the production of fermentation ethanol, is to reduce the energy requirements.

The energy balance of ethanol can be computed in many different ways (368, 374, 375). Frequently, the net energy ratio (NER) is used to indicate the efficiency of converting various substrates (sugar cane, beet, cassava, etc.) to ethanol (375, 376). The NER can be defined as the ratio of the total energy content of ethanol to the energy input. In producing ethanol from biomass, energy is expended directly in the form of fuel and indirectly in the form of equipment and labor. In fact energy is required in all four major areas in an alcohol plant: feed stock generation, preparation, fermentation, and product recovery. It is often argued that the NER of ethanol from biomass is unfavourable (368). However, if one considers the potential energy generated from waste streams in the form of biogas, and by-product credits from lignin, chemicals, etc. (Figure 1), ethanol from lignocellulosic biomass may allow an alcohol plant to be totally energy self sufficient and cost effective (377-384).

3. Microbial Features for Ethanol Production

Greater than 95% of the fermentation ethanol produced worldwide employs the yeast *S. cerevisiae* and its related species (340). However, numerous other microorganisms are also capable of producing ethanol from sugars. This section will briefly compare the general microbial features of yeast and bacteria that make them suitable for ethanol production (summarized in Table 11). A more detailed review of the biology of ethanol producing microorganisms was recently published by Stewart and co-workers. (340). In addition, many other reviews on fermentation ethanol production have been published (385-389).

Several fungi such as *Fusarium oxysporum* are also capable of producing ethanol (390-392). However, because of their very slow metabolic rates and low ethanol yields, they are not promising for industrial fermentation (393). Consequently, there has been little research with them.

In the production of ethanol, the microorganism being employed should possess a number of important features (340): 1) good ethanol tolerance, 2) rapid fermentation rate, i.e., high productivity, 3) high fermentation efficiency,

4) broad substrate spectrum, 5) temperature tolerance, 6) appropriate flocculation and sedimentation characteristics, 7) osmotolerance, 8) genetic stability, 9) produce few by-products, 10) maintain high cell viability.

The fermentation of glucose to ethanol as carried out by yeast is an efficient, well developed, and relatively simple process. Yeasts convert hexoses to ethanol via the Embden-Meyerhof-Parnas Pathway, where, theoretically, 1 g of glucose will yield 0.51 g of ethanol and 0.49 g of CO_2 (340). The result is 2 moles of ATP originating from the breakdown of 1 mole of glucose to 2 moles of ethanol and 2 moles of CO_2. Compared to most bacteria, yeasts are relatively more alcohol tolerant; however, ethanol is usually toxic to the cell at levels between 8 and 18% (w/v) depending on the strain and metabolic state of the culture. The low pH during ethanol fermentations minimizes bacterial contamination problems.

S. cerevisiae is limited by the number of substrates it utilizes. Although it can rapidly ferment glucose, maltose, sucrose and fructose, it is unable to ferment xylose, starches, and cellulosics. Since some strains of *S. cerevisiae* can ferment xylulose well, some reports have been made on efficient xylose fermentation, whereby xylose is converted to xylulose by inclusion of glucose isomerase in the medium (394, 395). Attempts to obtain suitable expression of cloned bacterial glucose isomerase in *S. cerevisiae* have been unsuccessful (396). Furthermore, it was recently shown that several yeasts such as *Pachysolen tannophilus* and various *Candida* species can ferment xylose (395-400). However, several problems still inherent in xylose fermentation, prevent pentose fermentation by any industrial process. Slapack and co-workers (401, 402) recently have shown that good xylose fermentors perform poorly in spent sulfite liquor because they are more sensitive to inhibitory substances such as acetic acid, than are poor xylose fermentors.

Various processes for the production of alcohol from biomass employ the yeast *S. cerevisiae*. However, this is only after the cellulose is first hydrolyzed by acid or enzyme, an extra cost factor. *Trichosporon* species of yeast show some evidence of minimal cellulase activity (403). Despite attempts of cloning and/or expression of cellulase genes into yeasts (404-406), there are no yeasts presently reported that can efficiently ferment cellulose to ethanol.

The formation of ethanol as a major product is also widespread among both anaerobic and facultative mesophilic bacteria (Table 12). Even aerobic bacteria like *Pseudomonas* can produce ethanol if it is grown anoxically (11). Esser and Karsch (393) recently assessed the advantages and disadvantages of bacterial ethanol production. By far, the only promising mesophilic bacteria for ethanol production are strains of *Zymomonas mobilis*, the species which has been traditionally used in tropical countries to make pulque and alcoholic palm wines (408, 411).

From extensive reviews (408, 411-414) it is evident that *Z. mobilis* possesses a number of interesting properties relevant to efficient alcohol production. It is capable of fermenting high concentrations of glucose (up to 40% w/v) to ethanol in batch and continuous cultures (411, 415). Furthermore, its specific rate of glucose uptake and of ethanol production can be even higher than those found with yeasts (361, 413). The reason for the high ethanol productivities (up to 120-210 g/ℓ/h) (361, 414, 416), is due to its unique metabolic pathway. *Zymomonas spp.* employ the Entner-Doudoroff Pathway in which one mole of ATP is formed per mole of glucose (415). As a result of lower metabolic energy for growth, lower biomass is produced than for yeasts (81, 408). This can sometimes pose a problem (especially at low substrate concentrations), since obtaining sufficient biomass for fermentation may be time consuming and costly. Since *Zymomonas* grows anoxically, unlike yeasts they do not require the controlled addition of oxygen to maintain viability at high cell concentrations (413). They have high ethanol tolerance comparable to *S. cerevisiae* [batch 13% (w/v), continuous 8.5% (w/v)] (413). Genetic manipulation of the procaryote *Zymomonas* should be simpler than for yeasts (412, 413, 417).

A number of processes employing *Z. mobilis* for alcohol production were recently developed: (i) Biohol Developments, a joint venture between George Weston Ltd. and the Ontario Energy Corporation, Toronto (418, 419); (ii) University of New South Wales (411-415). However, there are still no large-scale operations using *Z. mobilis* for fermentation ethanol. *Z. mobilis* strains exhibit a very narrow substrate utilization spectrum and have stringent nutrient requirements (408). To date, all known strains use only glucose, fructose, and occasionally sucrose (408). Unlike yeasts, the bacteria require pH values above 5, which sometimes necessitates extra costly pH control (420). Since this pH is more conducive for growth of contaminants, thorough sterilization of the media is required (81). Lower ethanol yields can be obtained from some strains as a result of the production of levan polymers from sucrose and acetic acid as a minor by-product. Most strains are poorly flocculent and this poses difficulties in cell recycle systems in continuous fermentations; however, flocculation mutants have been isolated (419, 421).

Karsch and co-workers (81) recently conducted a study comparing ethanol production by *Z. mobilis* and *S. cerevisiae*. The study confirmed previous investigations that *Zymomonas* produced more ethanol and less biomass than *Saccharomyces*. However, from an economic point of view, *Saccharomyces* was still preferred, mostly because of the necessity to sterilize the culture media for *Zymomonas*.

Rogers and co-workers in Australia (411, 415) have studied the effect of temperature on ethanol production by several strains of *Z. mobilis*. The optimum temperature for fermentation was determined to be ~30°C. Although strains fermented up to 40°C, cell viability, biomass, and ethanol yields were all decreased. Research however is proceeding to isolate more temperature tolerant strains (411). Viikari *et al.* (422) reported strains of *Z. mobilis* which fermented equally well at 37°C, while production at 40°C and 45°C were inhibited by ~30 and 60%, respectively, of that compared to 37°C. From a survey, Swings and De Ley (408) reported that only 5% of *Zymomonas* strains grew at 40°C. Chase and co-workers (423) have recently initiated a study to select for thermotolerant strains of *Zymomonas*. It should be realized however, that growth of mesophilic bacteria at higher temperatures, (like yeasts), can depend on various environmental and physiological factors (424). The addition of lipid-protein complex was not effective for enhancing the ethanol tolerance of *Z. mobilis* (420), as it was for yeasts (425). Although processes for the simultaneous saccharification fermentation (SSF) of starch were successful (426), SSF processes on cellulose, conducted with *Z. mobilis* were not (427, 428). As seen with other microorganisms, it was recently reported that ethanol significantly lowers the thermotolerance of *Z. mobilis*, by acting on the membrane (417, 429-431). It has also been reported that *Z. mobilis* fails to alter its fatty acyl composition in response to various ethanol concentrations (432). The significance of this will be discussed in Section IX, 2a.

Thermophilic bacteria have been reviewed in the past (39, 433). Thermophilic ethanol producing bacteria have steadily been gaining more attention as evidenced by the number of recent reviews (11, 13, 14, 27, 44, 46, 48, 59, 82, 139, 434-443). Cohn (444) isolated a facultative thermophile in 1875, while a strictly thermophilic bacterium was first isolated by Miquel (445, 446) from the Seine River and from feces, in 1879 and 1888, respectively. One of the most attractive features of thermophilic ethanologenic anaerobic bacteria is that they can metabolize a wide variety of substrates (Table 13) (11, 27, 59, 441, 447-452). In addition, some strains have: favourable doubling times, broad pH and temperature ranges, and produce ethanol in good stoichoimetric yields (Table 14) (11, 44, 59, 139, 434, 435, 441, 447-456). However, their low ethanol productivities and/or ethanol tolerances, make them presently less suitable than yeasts or *Zymomonas* for ethanol production. Specific properties and advantages of these thermophilic ethanologens will be discussed in detail in subsequent sections.

V. GENERAL PROPERTIES OF THERMOPHILIC ETHANOL PRODUCING BACTERIA

1. Anaerobic Non-Spore-Forming Bacteria

a. *Thermobacteroides acetoethylicus*

T. acetoethylicus (strain HTB2), a non-spore-forming obligate anaerobe was recently isolated by Zeikus *et al.* (457) from an algal mat in Octopus Spring located in Yellowstone National Park in the U.S.A. The taxonomic characteristics of this bacterium differ significantly from other obligate caldoactive fermentative anaerobes (458). Although cells stain gram negative, the cell wall architecture was not typical of gram-positive or gram-negative bacteria. This organism has a double-layered cell envelope structure. The very short generation time of 25-30 minutes distinguishes this species as one of the most prolific thermophilic anaerobic bacteria. The absence of detectable cytochromes suggests that energy conservation is via substrate level phosphorylation alone. This opens up the possibility to produce ethanol quite economically i.e., without wasting sugar for biomass production using stationary cells (82). It was observed that yeast extract is a fermentable substrate and growth was inhibited by O_2, NaCl, and some antibiotics. *T. acetoethylicus* is the only thermophilic ethanologenic bacteria which does not produce lactate as a by-product (82, 458). However, it produces nearly equal amounts of ethanol and acetate from glucose, independent of the medium supplementation.

b. *Thermoanaerobium brockii*

This asporogenous, gram positive, obligate anaerobe isolated by Zeikus *et al.* (449) is one of the most predominant anaerobic thermophiles in volcanic environments (457). *T. brockii* has a monolayered cell wall and cells appear to divide by a constrictive mechanism (449). Growth is inhibited by oxygen, penicillin, cycloserine, streptomycin, tetracycline, and chloramphenicol.

Zeikus *et al.* (449) found that it required ~0.05% yeast extract for growth. With this amount of yeast extract and 0.5% glucose, 0.95 mol of ethanol is formed per mol of glucose (459). However, in the presence of 0.1% yeast extract, a shift is observed toward a higher yield of lactate with less ethanol.

Sonnleitner *et al.* (460) recently investigated the growth behavior of this organism in both batch and continuous culture. They found that growth of *T. brockii* was substrate inhibited at concentrations exceeding 1.5% (w/v) glucose. The effect of high substrate concentration was different in batch than in continuous culture. In batch cultures high glucose concentrations resulted in acetic acid production while in chemostat cultures, virtually no products were formed. At low substrate concentrations higher growth rates were obtained in the chemostat. This may be due to the fact that actual substrate concentrations are always lower in chemostats.

Yeast extract requirements were shown to be exceptionally high; (60% of the glucose concentration used). Cultures with sufficient glucose and limited yeast extract were not good inocula (460). The growth factors in yeast extract are still unidentified. Similarly, glucose-depleted cultures could not be used as inocula. In batch culture, growth always stopped before glucose was fully depleted. Upon depletion of glucose, the cells became non-viable and yeast extract could not serve as an energy source. The maintenance requirements could be met only by glucose and not by yeast extract.

The observed higher ethanol/acetate ratio with an increase in pH, coincided with the higher pH optimum of its alcohol dehydrogenase (ADH) (460). Both Sonnleitner *et al.* (460) and Zeikus *et al.* (449) concluded from their studies that *T. brockii* is not an ethanol producer that can compete with mesophiles. Under all conditions tested, the maximum specific ethanol formation rate was only 0.7 g/g/h (460). Since it can use lactose at high temperature it may be suitable for the exploitation of whey (449).

The pathways from pyruvate to final products have also been determined by examining enzymes in cell free extracts (461). Pyruvate is converted to lactate by a NAD-dependent-L-lactate dehydrogenase (LDH), which is allosterically activated by fructose diphosphate (FDP) (441). It is thought that during growth on starch, a low concentration of intracellular FDP is responsible for the slower fermentation rate of starch versus glucose (462). Acetate is produced in reactions catalyzed by a ferredoxin-dependent pyruvate dehydrogenase, phosphotransacetylase, and acetate kinase.

T. brockii has both NAD- and NADP-dependent ADH's. The NAD linked enyzme is very sensitive to oxygen (462). The reactions catalyzed by the two ADH's are reversible and together they constitute a NADH-NADP transhydrogenase system. The NADP-dependent ADH has been purified to homogeneity and appears to have properties distinct from those of previously described primary and secondary ADH's (463). This enzyme has a molecular weight of 150,000, and is made up of four identical subunits. Although it is slowly inactivated at 86°C, it is stable at 65°C. The broad substrate specificity of this ADH allows *T. brockii* to oxidize secondary alcohols to ketones faster than primary alcohols to their corresponding aldehydes (462). In fact, growth of *T. brockii* in media containing acetone and yeast extract has been observed.

Recently, studies on the metabolic pathways of this organism have shown that ethanol yields can be improved by altering the normal electron flow during saccharide fermentation or by altering LDH activity. This will be considered in some depth in Section IX, 1b, i.

c. *Thermoanaerobacter ethanolicus*

Two strains of *T. ethanolicus* [originally named *Thermoanaerobium ethanolicum* (464)] have recently been isolated by Wiegel and Ljungdahl (451) from the hot springs in Yellowstone National Park. Strain JW200 has been designated the neo-type strain of *T. ethanolicus*. *T. ethanolicus* differs from other *Clostridium* and *Thermoanaerobium spp.* in its morphology and cell wall structure. This bacterium ferments 1% (w/v) glucose or starch nearly quantitatively to ethanol similarly to some yeasts and *Zymononas* (up to 1.95 mol of ethanol per mol of glucose) (59, 465). Unlike other thermophilic anaerobes which grow optimally at neutral to alkaline pH, it displays an equivalent growth rate on glucose at pH 5.8 to 8.5. Furthermore, the fermentation balance does not vary much over this pH range nor in the temperature range of 40 to 77°C (59). Contrary to these results, Kannan and Mutharasan (466) recently observed that an initial pH of 8 resulted in a higher specific growth rate and concentration of ethanol for *T. ethanolicus*, as compared to an initial pH of 5-7.5.

Although growth on xylose is as rapid as glucose [~120 minutes doubling time (D_t)], the pH range is much more restricted; xylose does not support significant growth above pH 7.4 and below 6 (452). The stoichiometry is also different; with 1% (w/v) xylose, up to 1.4 mol ethanol per mol of xylose is formed, whereas, it decreases even further with higher xylose concentrations (452, 465).

It can be seen in Table 13 that *T. ethanolicus* can use a wide range of substrates. When growing in media containing 10% (w/v) glucose or less, ethanol is the main product; however, when glucose concentrations exceed this amount, only 0.5% (w/v) ethanol is obtained because of lactic and acetic acid formation (59). Furthermore, a pH of $\geq$7.5 favours lactate production. Although this bacterium grows in solutions containing up to 20% (w/v)sugars or starch, fermentation ceases when the ethanol level reaches ~0.6% (w/v). Growth is slightly inhibited by 0.2 M acetate, 0.1 M lactate, 0.5 M ethanol, and 10% (v/v) hydrogen in the gas phase (59). Without any adaptation, total inhibition of growth occurs with 0.5 M acetate, 0.4 M lactate, or 1.3 M ethanol (450). However, with adaptation and selection techniques, mutants have been isolated that can tolerate up to 10% (v/v) ethanol (441). Both the shifts in product formation and the inhibition of fermentation appear to be related to metabolic control at the enzyme level and will be discussed subsequently (Section IX, 1b, iii). *T. ethanolicus* also contains two different ferredoxins and a rubredoxin (464).

Although the wild type can grow in substrate concentrations up to 25% (w/v), and tolerate 10% (v/v) ethanol, it produces ethanol in good stoichiometric ratios only at a substrate concentration of 1% (w/v) or less (441). However, *T. ethanolicus* strain JW200 (Fe_4) produces ethanol at good stoichiometric ratios from up to 4% (w/v) glucose, 3% (w/v) xylose, and 6% (w/v) soluble corn starch or cellobiose (452, 465). This strain produced 1.3 and 3% (v/v) ethanol from 3 and 6% (w/v) xylose and soluble corn starch, respectively (441). Other mutants of *T. ethanolicus* [JW200 Fe(3), Fe(7), Large, etc.) have been described and protected by a patent (467).

T. ethanolicus and its mutants can utilize xylan which will be discussed subsequently (Section VII, 4). Wiegel *et al.* (170) have not encountered influence on *T. ethanolicus* fermentation by inhibitory products expected from steam-mediated processes during hydrolysis of biomass. Furthermore, the addition of phenol, vanillin, syringenic aldehyde, furfural, or hydroxymethylfurfural (0.5% w/v) had no significant inhibitory effect on *T. ethanolicus* fermentations (170). *T. ethanolicus* can also ferment starch directly without the need of a prior hydrolysis to glucose and maltose (450). With xylose-utilizing yeasts and fungi, glucose represses xylose utilization (390, 468); however, with *T. ethanolicus*, both carbohydrates are used concurrently (465). Glucose supplied in equal amounts with either cellobiose or starch, inhibits the breakdown of these saccharides until its concentration decreases to ~20 mM. The cellobiase from *T. ethanolicus* JW200 has recently been isolated and characterized by Mitchell and co-workers (469). Although this enzyme has a strong affinity for cellobiose, it is strongly inhibited by its end product glucose [eg., ~2.5% (w/v) glucose inhibited activity].

The highest ethanol formation rate calculated from a growing culture on 2.3% (w/v) xylose was below 10 mM per ℓ per hour (470). Therefore, the rate of 30 mM ethanol per ℓ per hour for resting cells of *T. ethanolicus* on xylose appears promising (470). With resting cell suspensions, up to 60 mM per ℓ per hour of ethanol was produced from glucose (470). It should be noted however, that these ethanol tolerant mutants were selected only in the presence of starch in the medium. Results suggest that the selection of ethanol tolerant mutants in the presence of carbohydrate mixtures, would be more desirable to successfully employ *T. ethanolicus* for fermentation of mixed substrates (465). The use of *T. ethanolicus* and its mutants in mono-culture or co-culture with *C. thermocellum* has been protected by several patents (467, 471-474).

The exact nutritional requirements for *T. ethanolicus* have yet to be established. The medium that is presently used to grow *T. ethanolicus* is complex containing many vitamins, amino acids, and metals (455). One key nutritional requirement is zinc, which is a component of the ADH. The bacterium requires yeast extract, which cannot be replaced by tryptone, casein hydrolysate, beef extract, or ashed yeast (450). Kannan and Mutharasan (466) recently found a simpler medium than the original one described, and this was reported to provide the essential nutrients for growth and ethanol formation. A systematic study of ethanol yield under various yeast extract levels indicated that the essential growth factors were in sufficient supply at a yeast extract concentration of 0.6% (w/v), up to a glucose concentration of 5% (w/v). Unfortunately, attempts to substitute yeast extract with corn steep liquor did not yield comparable results (466).

2. Anaerobic Spore-Forming Bacteria

a. *Clostridium thermosaccharolyticum*

C. thermosaccharolyticum was originally isolated by McClung (475) in 1935, from spoiled canned foods. A bacterium very similar to this *Clostridium* was later described by Mercer and Vaughn in 1951 (476). The cultures were given the name *Clostridium tartarivorum nov. spec.*, because of their unique ability to ferment tartrate (476). Based on DNA homology studies, *C. tartarivorum* has been regarded as the tartrate-fermenting biotype of *C. thermosaccharolyticum* (477).

Taxonomically characterized strains optimally ferment a wide variety of carbohydrates as seen in Table 13. Sjolander (478) found that this species ferments glucose and xylose to acetate, butyrate, lactate, CO_2, and H_2, during growth. However, resting cells and cells in the sporulation phase (non-dividing or elongated) produce considerable amounts of ethanol (454, 479). Several factors discussed extensively by Hsu and Ordal (447, 479-482) influence the development of the elongated cells and sporulation. Good sporulation was induced with *C. thermosaccharolyticum* when the supply of carbon source was reduced but not absent (481). Conditions of restricted growth were achieved by the slow feeding of glucose (447). However, excess glucose repressed sporulation and ethanol production as little ADH was formed (447, 482).

Hoffman *et al.* (483) observed that when cell division of *C. thermosaccharolyticum* was interrupted, cells elongated and converted acetate to ethanol. Landuyt *et al.* (484, 485) monitored morphological changes and metabolic products produced when cell division was interrupted by use of a continuous dilution culture. By continuously diluting the cultures with fresh medium and transferring the cells after one generation of growth into an environment with reduced substrate concentration and increased culture volume, it was possible to maintain a low level of readily utilizable carbon source. Furthermore, by these conditions, it was possible to maintain a slow growth rate and a precise physiological state whereby the cells elongated extensively and produced ethanol. However, the eventual appearance of multiseptations along the filaments in many cells and the reversal to vegetative multiplication, resulted in an abrupt decrease in ethanol formation and a shift to acid formation. Their experiments strongly indicated that morphological changes may be responsible for a metabolic shift from acid to ethanol fermentation and pH was important (485). The transition is not just a matter of reducing pre-existing organic acids (486), but rather a consequence of gene expression that accompanies early stages of cell division or sporulation (484). Although ethanol concentrations were much too low for a viable process, they have recently filed for a U.S. patent on this novel process (Hsu - personal communication).

Lee and Ordal (454) have demonstrated the presence of the glycolytic enzymes in *C. thermosaccharolyticum*. All of these enzymes except for pyruvate kinase show less susceptibility to heat inactivation than the corresponding enzymes from mesophiles (487). Fructose biphosphate aldolase has been partially purified by Barnes *et al.* (229). This enzyme has a requirement for divalent cations such as Co^{+2}. It has a sharp pH optimum of 7, is active at 70°C, and it is stimulated by NH_4^+ or K^+. Triosephosphate isomerase has also been purified from *C. thermosaccharolyticum* (488). This enzyme is stable at 55°C, with a pH optimum between 7.5 to 9.2. It also contains a greater amount of proline than its mesophilic counterpart. Resting cells of *C. thermosaccharolyticum* employ enzymes of the hexose monophosphate shunt pathway (454).

C. thermosaccharolyticum was initially not regarded as a potential producer of industrial ethanol since the wild type was inhibited by ethanol concentrations $\geq$1% (w/v). Although mutants have been selected which can tolerate up to 5% (w/v) ethanol (Section IX, 2a), these isolates still produce only low amounts of ethanol. This is discussed in subsequent sections.

b. *Clostridium thermohydrosulfuricum*

This *Clostridium* was first isolated from extracts obtained in an Austrian sugar beet factory by Klaushofer and Parkkinen in 1965 (489). Several of its properties allow this organism to be as ubiquitous as *B. stearothermophilus*, the facultative anaerobic counterpart to this *Clostridium* (11). *C. thermohydrosulfuricum* is found in a variety of mesophilic and thermophilic environments from many sources (eg. mud, hot springs), and locations (eg., U.S., Europe) (11, 46, 455, 457). The species name *thermohydrosulfuricum* indicates its ability to grow at high temperature and produce large amounts of hydrogen sulfide from sulfite and thiosulfate, but not from sulfate (441). *C. thermohydrosulfuricum* strain E100-69 isolated by Hollaus and Klaushofer (9) was originally considered the neotype for this species (477). However, Wiegel's *C. thermohydrosulfuricum* strains JW102 (455), and JW200 (448) and Zeikus' *C. thermohydrosulfuricum* strain 39E (457) are also now well characterized.

Variations in stoichiometry range usually from 0.5 to 1.6 moles of ethanol formed per mole of glucose utilized (11, 448). Recently, a higher amount of ethanol (1.95 moles) was reported by Ng *et al.* (456) using *C. thermohydrosulfuricum* strain 39E.

Although *C. thermohydrosulfuricum* grows from pH 4.8 to 9.7, the pH optimum is rather narrow, and the initial and final pH are important parameters affecting ethanol production (448, 455). A starting pH of 5.2 or less does not result in any ethanol formation. Reasonable yields of ethanol were only produced when the pH was allowed to drop from 7.2 or 7.5 to 6.9 or less during fermentation (11). Low yields of ethanol were obtained with growing and resting cells when the pH was kept constant. The pH effect has yet to be explained.

Opposite to the pH effect, ethanol was produced at all growth temperatures without significant changes in the amount of other by-products. In fact the growth rate of *C. thermohydrosulfuricum* was the same at 62°C as it was at 55°C (59). Although the reason for this is unknown, it is conceivable that it reflects a transition temperature above which new proteins have to be synthesized for this bacterium to function. Evidence for such a concept has been reported by Haberstich and Zuber (490).

Preliminary results indicate that in *C. thermohydrosulfuricum*, the synthesis of some proteins is turned off at high temperatures, whereas the synthesis of others is turned on. Ljungdahl and co-workers (59) found fewer proteins in cells grown at 70°C in comparison with those grown at 50°C. However, some proteins were much more noticeable in cells grown at the higher temperature.

C. thermohydrosulfuricum requires yeast extract for growth, and the yield of cells depends on the amount of yeast extract supplemented to the medium (11). Resting cells also require yeast extract for higher ethanol yields; however it was shown that the yeast extract was not converted to ethanol (491).

Although *C. thermohydrosulfuricum* does not grow in the presence of oxygen, it is relatively tolerant to heavy metal ions and anions normally found in sewage, oxygen, acid or base shocks, and vacuum (11). However, products formed during fermentation are inhibitory (448, 455). High partial pressure of hydrogen, inhibits growth of *C. thermohydrosulfuricum* and decreases the fermentation rate (139).

Xylose metabolism is associated with xylose isomerase, xylulose kinase, and subsequent transformation of xylulose 5 phosphate into glyceraldehyde

3-phosphate which feeds into the glycolytic pathway (27). Zeikus *et al.* (27) examined growth of *C. thermohydrosulfuricum* on xylose in continuous culture under carbon and nitrogen limitation. The ethanol product ratio was not significantly altered by the xylose concentration supplied in the chemostat. Under nitrogen limitation, the yield of ethanol was low and lactate high, whereas the opposite occurred under carbon limitation. The results of glucose fermentation in a chemostat culture demonstrated that increasing the dilution rate significantly increased the lactate product ratio under both carbon or nitrogen limited conditions. This data is consistent with a mechanism for control of end products similar to that found in *T. brockii*, such that the less rapidly metabolized substrates (xylose or starch) in the presence of limited nitrogen, decrease the intracellular concentration of FDP and increase ethanol yield in lieu of lactate (Section IX, 1b, i).

Mancuso *et al.* (62) recently conducted a study investigating the physiological parameters affecting xylose fermentation with *C. thermohydrosulfuricum*. They found that environmental parameters such as pH and by-product concentrations had only a slight effect on the ethanol yield. However iron was important for ethanol fermentation and they recommended that ferrous sulfate (2 mg/ℓ) should be included in the medium in future work. Furthermore, their results indicated that methionine was very important for this organism and it could be met by vitamin B_{12}. *C. thermohydrosulfuricum* was gradually adapted to high concentrations of ethanol [up to 4.2% (w/v)]. However, the ethanol tolerant cells produced more lactate and acetate and ~35% less ethanol than the non-adapted cells. The shift in the distribution of fermentation products with ethanol tolerant adapted strains, may have been related to changes in membrane structure. Possibly, intracellular enzymes were affected by the highly fluid membrane in such a manner that organic acid formation was increased in favor of ethanol production (62). Mancuso and his colleagues concluded from their study that this bacterium was not economically feasible for converting sugars to ethanol.

Lovitt *et al.* (492) also obtained an ethanol tolerant isolate of *C. thermohydrosulfuricum*. They partly attributed ethanol tolerance in this isolate to be due to a unique membrane lipid composition containing C_{30} dicarboxylic acids. Since the wild type strain could readily tolerate >4% (w/v) methanol and acetone, the basis for ethanol intolerance was not due to solvent effects on membrane integrity, but rather a regulatory problem (492). The unique membrane composition of the wild type (no free fatty acids present) was thought to be responsible for its high solvent tolerance (Zeikus - personal communication).

An important feature which distinguishes *C. thermohydrosulfuricum* from other glycolytic thermophilic *Clostridia* is the fine structure of the outer layer of their cell wall (493). The surface layer in these bacteria has arrays of subunits which consist of glycoproteins (494, 495). In *C. thermohydrosulfuricum*, the subunits are arranged hexagonally, whereas in other *Clostridia* the subunits have a tetragonal symmetry (493).

An NADP-linked ADH as well as two different ferredoxins have been demonstrated in *C. thermohydrosulfuricum* (464, 496). The ADH reversibly reduces acetaldehyde to ethanol. However, it has a broad substrate spectrum and is more active towards secondary alcohols or ketones than towards primary alcohols and aldehydes.

Spore formation occurs easily on glucose agar medium when cells grown above 62°C are allowed to cool to temperatures below 55°C (455). The spores are very heat resistant and they commonly have a D_{10} time (time of reduction to 10% of cells surviving) of 2-5 minutes. One strain, *C. thermohydrosulfuricum* E-39 displayed a D_{10} value of 11 minutes at 121°C (497). This is high in comparison to the value of 3 minutes for *B. stearothermophilus*, which is commonly used as the standard to judge autoclaving procedures for sterilizing microbiological materials (498). In contrast, vegetative cells of *C. thermohydrosulfuricum* are rapidly killed at 80°C (D_{10} time of 30 seconds). The higher degree of spore heat resistance of this *Clostridium* was largely due to the thickness of its spore cortex (497). Heat resistance of clostridial spores was shown to be proportional to the ratio of spore cortex volume to cytoplasmic volume (497).

c. *Clostridium thermocellum*

The first mention of bacteria capable of fermenting cellulose at high temperatures (~65°C) comes from Macfayden and Blaxall in 1896 (499). As early as 1912, Pringsheim (500) demonstrated the presence of an anaerobic cellulose-degrading enzyme in a culture fermenting at 55°C. Studies in the 1920's on the industrial utilization of cellulosics were conducted by Lymn and Langwell (501) who obtained several patents on the thermophilic fermentation of cellulose. A complete history of thermophilic anaerobic cellulolytic bacteria is given in reviews by Mcbee (502) and Duong *et al.* (503).

A species of *C. thermocellum* that ferments cellulose to ethanol was first described by Viljoen *et al.* in 1926 (504). Until the 1940's, all efforts to obtain stable pure cultures of thermophilic cellulolytic bacteria were unsuccessful (505-507). However, Hungate's (508) methods permitted isolation of pure cultures of *C. thermocellum* by McBee (453) in 1948. *C. thermocellum* is the best characterized anaerobic cellulose degrading bacterium. It can be isolated quite easily from nearly all decaying organic matter (139).

A second type of thermophilic cellulolytic bacterium was isolated by Enebo (141, 509, 510) during his studies of cellulose degradation. Unlike McBee's strain, *C. thermocellulaseum* was able to utilize glucose, fructose, and mannitol. Since more recent isolates of *C. thermocellum* also differ in their substrate utilization abilities, *C. thermocellulaseum* is now considered to be a derivative strain of *C. thermocellum* (503). Hernandez (511) recently evaluated four strains of *C. thermocellum* for ethanol production. *C. thermocellum* strain ATCC 27405 was found to have the fastest growth rate and was the best ethanol producer; however, it failed to grow on xylose (512). It is noteworthy that McBee's (453) strain 157 which was reported to use xylose is the same as ATCC 27405.

All *C. thermocellum* strains are able to ferment cellobiose and cellulose. However, with the exception of a few strains, the difference seems to be in the ability to assimilate glucose or xylose (513-516). Lee and Blackburn (517) reported that *C. thermocellum* strain M7 was able to utilize cellulose, cellobiose, glucose, sucrose, galactose, raffinose, lactose, ribose, rhamnose, trehalose, dextrin, starch, salicin, inositol, and sorbitol. Most strains of *C. thermocellum* are quite similar in that they produce ethanol, acetate, lactate, and CO_2/H_2, and they are inhibited by the same products eg., 5% (w/v) ethanol, etc. As far as is known, all *C. thermocellum* strains produce ethanol from cellulose from 0.3 to 1 mole of ethanol per mole of glucose equivalent (502, 504, 515, 517). This low ethanol tolerance is one of the major problems for industrial alcohol production, and will be discussed in depth subsequently (Section IX, 2a). Figure 2 illustrates the fermentation characteristics of *C. thermocellum* during growth on cellobiose. It can be observed that the fermentation products are formed as a direct consequence of growth, but these products also continue to increase in concentration after growth ceases.

The presence of other pentose utilizing *Clostridium spp.*, which form closely associated stable mixed cultures with *C. thermocellum*, can probably account for the reported xylose fermentation capabilities of *C. thermocellum* strains described in earlier investigations. In this regard, many cultures of *C. thermocellum* thought to be pure, have been shown to be mixed cultures with time. *C. thermocellum* strain S-8, capable of growing on glucose, xylose, cellulose, and cellobiose, was isolated during an ethanol adaptation tolerance program (434). Even after 8 transfers on xylose, Wang *et al.* (434) found that this strain still exhibited cellulolytic activity. Careful biochemical and morphological examination have led this group to believe that it is a stable mixed culture of *C. thermocellum* and *C. thermosaccharolyticum*. Similarly, *C. thermosaccharolyticum* strains HG2 and ZC were isolated as contaminants from *C. thermocellum* strains H1, and LQ8, respectively (439, 515, 518).

The ability of *C. thermocellum* strains to metabolize glucose as the sole carbon source has been the subject of much controversy in the literature (438, 515, 519). Some strains of *C. thermocellum* are able to utilize glucose if there is initially 0.5-0.6% yeast extract in the medium; however this is often after a lag of ~12-30 hours (512-514, 519, 520). After a few transfers on glucose, these cultures continue to grow, with a small amount of yeast extract (~0.02%) present in the medium, but with less of a lag time.

Patni and Alexander (513, 514) reported that glucokinase was an inducible enzyme and that yeast extract stimulated its production thereby facilitating glucose metabolism in *C. thermocellum*. Gomez and Hernandez (520) showed that cultures that had not been adapted to growth on glucose were unable to transport this sugar, whereas glucose-adapted cells could. Preliminary experiments by Hernandez (521) showed no clear evidence for the existence of a phosphoenolpyruvate phosphotransferase system for the transport of D-glucose, as was previously found for mannitol and fructose (514). Although further evidence is still required, the uptake of glucose by glucose-adapted cells appears to be mediated by an ATP-dependent permease transport system (521). The existence of such a system can explain the observation that glucose is energetically less favourable than cellobiose in sustaining growth of *C. thermocellum* (521).

Patni and Alexander (514) demonstrated the presence of mannitol phosphotransferase and mannitol 1-phosphate dehydrogenase, enzymes needed for mannitol fermentation. Lamed and Zeikus (461) reported an ammonium ion activated-NADP-linked malic enzyme and an unidirectional NADH-dependent oxaloacetate reductase in *C. thermocellum*. The malic enzyme has been purified and characterized (522). Forget (253) recently purified and characterized a heat stable eight iron ferredoxin isolated from *C. thermocellum*.

3. *Bacillus stearothermophilus*

B. stearothermophilus is the most studied thermophile. There is an extensive amount of information available on *B. stearothermophilus* due to its importance as a contaminant in the beverage, food, and canning industry. Studies with this *Bacillus* have led to important findings about general aspects of thermophily (molecular mechanisms, effects of nutrients, etc.). These will be dealt with in the appropriate sections.

The neotype strain was described by Donk (523) in 1920. Numerous strains have since been isolated and many unclassified *Bacillus spp.* used in studies of thermophily seem to be related to *B. stearothermophilus* (289, 524-528). Some of its basic physiology has been previously reviewed (44, 525). *B. stearothermophilus* is ubiquitous as it can be found in soil, mud, or water, in both warm or cold climates. It is easy to culture and grows optimally between 50-65°C. Its T_{min} and T_{max} are about 28 and 70°C, respectively (44). *B. stearothermophilus* can grow on a wide range of substrates: starch, glucose, xylose, fructose, maltose, mannose, casein, phenols, cresols, catechols, acetate, succinate, glycerol, and paraffin (226, 525, 529, 530).

Jung *et al.* (531) showed there were metabolic differences in *B. stearothermophilus* cells grown at 55° and 37°C. At 55°C there were high activities of glyceraldehyde 3-phosphate dehydrogenase (GPDH) and ADH, whereas, at 37°C no ADH was detected and GPDH activity was low. Furthermore at 37°C, TCA cycle enzymes were more active, and slow growth was found under anaerobic conditions. These workers hypothesized that at 37°C, energy was obtained mainly by aerobic respiration, while at 55°C, both respiration and fermentation pathways could be employed. The preferred pathway used, would depend on the oxygen content and the phase of growth (531 [illegible] might have been predicted, Atkinson and co-workers (532) showed that this *Bacillus sp.* was capable of producing ethanol at temperatures >55°C. However, they concluded that this bacteria was not suitable for alcohol production.

Hartley and his colleagues (369, 443, 533) at Imperial College, in London, England, found it possible to metabolically steer this bacterium to produce high yields of ethanol by genetic manipulation and optimization of fermentation conditions. After assessing the economics of various proposed schemes for continuous ethanol removal, it appears they have designed a viable process. The ingenious route they employed illustrates the multidisciplinary approach (i.e., physiology, biochemistry, genetics, and engineering) needed to make thermophilic fermentations economically feasible. For their studies, Hartley *et al.* (369, 443, 533) employed *B. stearothermophilus* NCA 1503. This facultative anaerobe utilized glucose, sucrose, maltose, xylose, and cellobiose. Its doubling time was approximately 30 minutes at 70°C and its growth rate was unaffected by ≤4% (w/v) ethanol.

Most facultative anaerobes including *B. stearothermophilus* metabolize pyruvate aerobically via pyruvate dehydrogenase (PDH) and the TCA cycle, in which, PDH converts pyruvate to acetyl CoA and then to ethanol (Figure 3). However, anaerobically the PDH pathway appears to be inactive probably because the NAD^+/NADH balance is unfavorable (369). The facultative anaerobes metabolize pyruvate anaerobically via a NADH-linked LDH to lactic acid, or pyruvate-formate lyase (PFL) to formate and acetyl CoA. The acetyl CoA can then form acetate via a phosphotransacetylase and acetate kinase, or ethanol via acetaldehyde dehydrogenase and ADH. This hypothetical pathway is illustrated in Figure 4. The NADH balance for the PFL pathway would be 1 mole of lactate, 1 mole of formate, 0.5 moles of acetate and 0.5 moles of ethanol per mole of glucose metabolized.

The good ethanol producers (yeast and *Zymomonas*) are known to produce 2 moles of ethanol per mole of glucose via a pyruvate decarboxylase pathway. It has been hypothesized that *B. stearothermophilus* gives high yields of ethanol via a PDH pathway (369). However, ethanol might also be obtained if this *Bacillus* had other enzymes such as an NAD-linked formate dehydrogenase. On this assumption, it was predicted that high yields of ethanol (comparable to yeast fermentation) could be obtained if it were possible to inactivate the PFL pathway, and activate the PDH pathway. With this in mind, Hartley and co-workers examined various strategies of accomplishing this (369).

Employing a novel approach using fluoropyruvate (369), an LDH negative mutant was selected. The stable mutant *B. stearothermophilus* LLD-15 formed no lactate, and yielded the ratio of products predicted from the new NADH balance (i.e., 2 moles of formate, 1 mole of acetate, and 1 mole of ethanol). Various mutational strategies to inactivate the PFL pathway in *B. stearothermophilus* LLD-15 were unsuccessful; presumably this enzyme is essential for anaerobic growth (369). Therefore these workers explored fermentation conditions which might switch on the PDH and switch off the PFL pathway. By manipulating culture conditions, they approached the target of 2 moles ethanol per mole of glucose, or 4 moles ethanol per mole of sucrose. They found that higher temperatures (up to 70°C), and increased sucrose concentrations [up to 5% (w/v)] favoured ethanol formation [up to ~2% (w/v) ethanol]. Furthermore, fermentation at pH 6.2 resulted in maximal ethanol to formate and acetate ratios. Figure 5 demonstrates the fermentation characteristics of *B. stearothermophilus* LLD-15 cultivated at 60°C in 5% (w/v) sucrose. Batch studies in 5 *l* fermentors using 5% (w/v) sucrose demonstrated that during the first 5-7 hours, the various by-products from the PFL pathway were formed. After this exponential growth phase, a linear phase of growth existed for about 18 hours. During this stationary phase, the PDH pathway predominated as sucrose was essentially converted to ethanol, i.e., acetate and formate production ceased. As might have been expected from the above, continuous culture resulted in lower ethanol yields as both pathways operated approximately to the same degree (369).

It is still uncertain why, when growth stops, the PFL pathway is switched off. It is not due to nitrogen source, nor to exhaustion or accumulation of metabolites (369). In attempting to find the switch for the putative PDH pathway, Hartley *et al.* (369), examined the effect of adding ethanol, acetate, or formate to the fermentor during the exponential phase of growth. Only when acetate and formate were added together, did growth stop and the switch to the PDH pathway occur. The reason for this is unknown, but it may be due to an uncoupling effect of these anions on the membrane, or due to difficulties in the excretion of these metabolites (369). Perhaps in the future it might be possible to obtain mutants that would act as if acetate and formate were already present in the media. One would think that *B. stearothermophilus* would perform well in a recycling process where little growth is necessary. However, the author is unaware of any recycling experiments performed.

Hartley *et al.* (533) discussed the economics of various proposed models of continuous processes for removing ethanol from *B. stearothermophilus* cultures during fermentation of 10% (w/v) cane juice; namely, vacuum (0.270 mm Hg) and CO_2 stripping. These processes were designed to reduce the ethanol to ~4% (w/v) in the liquid phase. Although their assessment was primarily comparative, they found production costs to be \$353/m^3 for the mesophilic yeast process, and ~\$295/m^3 for the thermophilic vacuum or CO_2 stripping process (based on 1980 cost estimates). Further predictable improvements with *B. stearothermophilus* would lower the thermophilic fermentation costs to \$263/m^3 (533). This savings of 14 or 25% of the production costs appears fairly attractive. Hartley is presently collaborating with the Indian Council for Scientific and Industrial Research in taking this process through development (Hartley - personal communication).

4. Other Thermophilic Bacteria

Many other thermophilic ethanol producing and/or cellulolytic bacteria have been identified. After proper identification and purification, many of these unidentified cellulolytic bacteria may eventually turn out to be various strains of *C. thermocellum*. Hu (534) studied the cellulolytic isolate C22225, a gram negative spore-former resembling *C. thermocellum*, but with an optimal pH for growth of 8.5. Hayashida *et al.* (140) recently isolated a new thermophilic ethanologenic cellulolytic *Clostridium sp.* from compost. Madden and co-workers (535) recently isolated and characterized *Clostridium papyrosolvens sp. nov.* an anaerobic, cellulolytic bacterium. *Clostridium stercorarium*, which has different enzymes than *C. thermocellum* (536), was reported to ferment arabinose, glycogen, lactose, maltose, mannose, melibiose, rhamnose, ribose, starch, and xylose (537). In contrast to *C. thermocellum*, it does not ferment amygdalin (537). Although its optimal growth and cellulase production rates occur at 65°C and pH 7.3 respectively, it has a slow doubling time of 16 hours. A characterization of the exoglucanase of this organism was recently made (538). Recent preliminary results of Ljungdahl *et al.* (59) demonstrate the existence of cellulolytic bacteria isolated from hot springs in Iceland, that are capable of fermenting cellulose in environments as extreme as pH 2 and at temperatures as high as 84°C.

Taya *et al.* (539, 540) isolated and studied a cellulolytic thermophilic anaerobic *Clostridium sp.* designated strain Tx. This strain was able to decompose most polymerized substrates present in natural cellulosics (eg., rice, straw, orange peel) with only a brief pulverization treatment. The optimal temperature and pH for growth were 60°C and pH 6-6.5, respectively. It fermented a wide range of substrates including glucose, xylose, cellobiose, cellulose, starch, pectin, and xylan. Strain Tx grew on a xylan medium even after 5 subcultures to exclude contamination of cellobiose from the original inoculum. This is in contrast to *C. thermocellum* which is inherently unable to utilize xylan and will not grow on xylan in a medium without cellobiose (516).

Researchers at the Solar Energy Research Institute (SERI), Colorado, enriched for xylan fermenting thermophilic anaerobic bacteria from acidic thermal springs (541-543). Two gram negative rods (OCB7H8 and FP7H10) which grew optimally at pH 5 and 55°C were isolated from these springs (542). These strains produced mainly ethanol from xylan, starch, and a variety of hexoses and pentoses. *Thermobacteroides saccharolyticum*, a xylan-fermenting asporogenic gram negative anaerobic bacterium has recently been isolated from Yellowstone National Park (543). It was reported to ferment xylan, starch, glucose, xylose, and cellobiose and had a temperature optimum of 60°C. Weimer *et al.* (544) recently described some new hemicellulolytic anaerobic thermophiles (similar to *T. acetoethylicus*) which had been specifically isolated on hemicellulose. These bacteria were reported to ferment xylan rapidly, ferment a wide range of carbohydrates, and contain plasmids (544). Wiegel and Puls (452) recently reported a new isolate LC2 that could grow on fractionated xylan of steamed birchwood.

C. thermohydrolyticum was reportedly isolated from a decomposing algal mat in Yellowstone National Park in 1981 (139). This species actively ferments saccharides including starch and pectin. Its doubling time on glucose is 2 hours, and it has a T_{opt} of ~60-62°C. The fermentation products of pectin are methanol, ethanol, acetate, lactate, and H_2/CO_2 (139). This thermophile was later renamed *C. thermosulfurogenes* (138). A characterization of the enzymes of *C. thermosulfurogenes* has recently been made (545). Further studies with this *Clostridium sp.* show that it has a similar membrane structure to *C. thermohydrosulfuricum* (i.e., contains C_{30} dicarboxylic acids) (Zeikus - personal communication). Furthermore, mutants have been recently isolated in Zeikus' laboratory which produce ~2-3% (w/v) ethanol.

Carreira *et al.* (546, 547) recently isolated and characterized three new thermophilic *Clostridium spp.* (RB-2, RB-2A, RB-3) (similar to *C. thermohydrosulfuricum*) from Icelandic Hot Springs. These bacteria were reported to ferment starch, cellobiose, maltose, glucose, mannose, ribose, xylose and pyruvic acid. They were also capable of growth at 23-63°C and pH 3-7. Other uncharacterized thermophilic ethanologenic bacteria have also been isolated (491, 548). Some mixed cultures of unidentified thermophilic bacteria were also reported to produce ethanol (549).

Although other thermophilic *Bacillus spp.* besides *B. stearothermophilus* exist (*B. subtilis, B. coagulans, B. acidocaldarius, B. caldotenax, B. caldolyticus, B. sphaericus, B. thermocatenulatus, B. thermoglucosidasius*) (34, 38, 433, 550), it appears that these have not been examined carefully for their fermentation ability. An unidentified *Bacillus spp.* similar to *B. stearothermophilus* which has not yet been isolated in pure culture ferments well in mixed culture with *Sporocytophaga sp.* (526, 528). Furthermore, Enebo (141) in 1951 reported some ethanol production from the thermophilic bacterium, *B. thermolacticus*.

VI. THERMOTOLERANT YEASTS

1. Growth at High Temperature

It becomes apparent that there are no true thermophilic yeasts. Although some species are definitely more thermotolerant than others, the maximum temperature for growth and fermentation depends on many factors (Table 1). There is even very little consistency in the literature as to how the optimum temperature should be defined. The optimum temperature can be defined as the temperature at which growth or fermentation rates or the cellular yield is the highest (551-553).

Although laboratories since the early 1900's have carefully examined the influence of temperatures on yeast survival and growth (554); little attention has been given to the role of temperature in fermentation processes. This is clearly evident from the fact that very few comprehensive reviews on this particular subject are currently available. Many yeasts that do exhibit good growth at high temperatures are unable to ferment at these temperatures. As early as 1914, the effect of high temperature on yeasts was examined and it was reported that the rate of alcohol production increased with temperatures up to ~40°C (555, 556). White and Munns (551) showed that the growth coefficient of *Saccharomyces spp.* increased steadily up to 30°C and then slightly up to 36°C, while the fermentation coefficient increased steadily up to 40°C. Krouwel and Braber (557) investigated ethanol production by *S. cerevisiae* at 30 and 39°C. They found that raising the temperature resulted in less growth and higher ethanol productivity. In fact, for most species the optimum temperature for fermentation was shown to be 5 to 10°C higher than that for optimum growth (174, 552, 558-560). However, Merritt (553) reported a distillery yeast (T_{max} of 35°C) which exhibited a maximal fermentation rate at 25°C. It has been suggested that ethanol production increases up to 40°C, largely in part due to an optimum activity of ADH near this temperature (560).

Many yeasts found in the digestive tract of animals (eg., *Candida slooffii* and *Candida krusei*) are referred to as thermophilic enteric yeasts (reviewed in 561-562). However, to the author's knowledge, these have not been tested for their fermentation ability. Some yeasts capable of growing up to 37°C are also often referred to as thermophilic (20), while others which have a T_{max} of >45°C are sometimes only implicated as being thermotolerant (21). Many yeasts, possibly quite capable of growing at higher temperatures, have not been cultivated under the optimum physiological conditions, or not even tested. These facts make it a difficult task to survey the literature on thermotolerant yeasts. Nevertheless, Table 15, consists of a partial list compiled from the available literature, of those species where growth was reported at temperatures $\geq$45°C.

Cooke (570) examined the ability of 2300 strains of 70 species of yeast for their ability to grow at 37°C in 3 days. The data indicated that growth temperatures varied for different strains of a given species. After examining 594 yeast strains, Vidal-Leira and co-workers (65) concluded that in most yeasts the T_{max} was fixed at the species level within a narrow range of temperatures. In 41 species of yeast where at least 5 strains were examined, intraspecific variation in T_{max} did not exceed 5°C (65). Minami *et al.* (571) screened for thermotolerant yeasts from soil samples in tropical regions and found 256 strains capable of growth at 40°C.

The highest reported growth temperature of a yeast is ~50°C (Table 15). Cooney *et al.* (565, 572) reported growth of *Hansenula polymorpha* on methanol at 50°C and in fact most strains of *H. polymorpha* are thermotolerant (564). As early as 1905, Johnson (163) described a yeast, *Saccharomyces thermantitonum* (similar to *S. cerevisiae*), that was capable of growth at temperatures up to 50°C. It was later reclassified as *S. willianus* by Lodder in 1952 (22, 573). Unfortunately, subsequent workers were unable to verify Johnson's results, the maximum temperature this species could be grown at by them was 38°C (22, 574). Another yeast, *Endoblastomyces thermophilus* was reported to grow at 48°C in a mixed culture with certain bacteria (22). However, in pure culture it had a T_{max} of only 44°C. Some *Candida* and *Kluyveromyces* species were also capable of growing at temperatures approaching 50°C (see Table 15).

Thermotolerant yeasts have been isolated from various sources such as sugar refineries (575), sugar cane compost (21), bovine rumen (576), tropical soil samples (571), etc. More studies should focus on isolating thermotolerant yeasts from thermal ecological niches as has been done with bacteria. An analysis of the yeast flora associated with desert cacti and flies has been made (577). Of 430 isolates from these sources, 97% had the ability to grow at 37°C. This high frequency of thermotolerant yeasts is not characteristic of yeasts from temperate or tropical regions (577). In fact, only 30% of 434 isolates listed in Barnett and Pankhurst (578), can grow at 37°C. Although in contrast to temperate and tropical yeasts isolated from flies, desert yeasts associated with cactiphilic *Drosophila* generally have low fermentation ability (577, LaChance - personal communication). Only ~20% of yeasts isolated from desert cacti are able to ferment glucose, as compared to ~70% of all yeasts in general (573). Ecological reasons are thought to account for the high thermotolerance and poor fermentation ability in yeasts associated with cacti (LaChance - personal communication). Desert yeasts are generally considered secondary invaders of necrotic cactus tissue, as they are often preceeded by microorganisms which are responsible for initiating the rot and carrying out the fermentation of sugars to alcohols and acids (577). This is exemplified by the fact that desert yeasts have high assimilation abilities for many alcohols and acids (577). Future isolations of thermotolerant yeast isolates from cacti should involve an enrichment for selective screening of yeasts with good fermentation ability. This can be simplified by use of Durham fermentation tubes. A particularly good source for such isolations might be sugar-containing compost.

2. Fermentation at High Temperatures

Although there are numerous references in the literature to yeasts having the ability to grow at temperatures ≥40°C, there have been very few reports of yeast fermentation at these high temperatures. In the author's view this is due mostly to lack of exploration in this area rather than to inherent physiological problems with the yeast. Tables 16-20 list those yeast strains from the available literature which were reported to ferment at temperatures ≥40°C. It is not appropriate to compare the fermentation performance of one

≥40°C. It is not appropriate to compare the fermentation performance of one yeast strain against another, since the strains were often tested by different criteria, in different laboratories, and under variable physiological conditions which were often not described in detail. Nevertheless, these lists could serve to indicate which strains are better candidates for future screening. Many of the studies pertaining to thermotolerant yeasts were conducted in tropical countries where cooling costs are high and their use would be even more advantageous. Other yeasts were screened at ≥40°C for their performance in simultanteous saccharification fermentation (SSF) processes, since the cellulase enzymes employed in such processes, have high temperature optima (Section VII, 3).

There has been one recent report of the screening of yeasts able to ferment at high temperatures. The Tate and Lyle Research Group in England (579, Hacking - personal communication) examined 55 yeast strains from 7 genera that were associated with good ethanol production and ethanol tolerance (Table 21). They screened these yeasts in flasks for their ability to ferment 14% (w/v) glucose at 37, 40 and 45°C. Ethanol yields of more than 50% of theoretical values (i.e., ≥4.5% v/v) were obtained with 28 strains at 37°C, 12 at 40°C, but none at 45°C. The 8 strains with the highest ethanol yields in shake flask cultures at 40°C were tested on 14% (w/v) glucose in 5-ℓ batch fermentations (Table 22). Of the strains examined, only *Saccharomyces uvarum* YSa85, *Saccharomyces cerevisiae* YSa86, and *Candida pseudotropicalis* YCa9 were able to match the minimum commercial targets set at ≥90% yield (≥8% v/v) in under 72 hours.

a. *Kluyveromyces spp.*

Since the Tate and Lyle study (579) consisted of only 55 strains, definitive conclusions about thermotolerance of the various genera studied cannot be made; nevertheless some trends were noted. It appears that *Kluyveromyces* strains were the most thermotolerant of the 7 genera examined. Of 12 strains examined, yields of more than 50% of the theoretical values were obtained with 8 strains at 37°C and 5 strains at 40°C (Table 21). Although ethanol yields were low, these 5 strains were also capable of growth and fermentation at 45°C (Table 22). Furthermore, they could not match commercial requirements in fermentors or flasks at 40°C. However, another strain of *Kluyveromyces marxianus* (discussed below) had an overall theoretical ethanol yield of 92% at 40°C (567). Despite good thermotolerance, *Kluyveromyces spp.* appeared to have lower ethanol tolerance at the high temperature, as indicated by the increased amount of residual sugar at the higher temperature (567, Hacking - personal communication).

Hughes *et al.* (567) studied the fermentation kinetics of a thermotolerant strain of *K. marxianus* in glucose-containing batch cultures over a temperature range of 30°C to 48°C. As seen in Table 23, the overall yield of ethanol declined in the upper temperature range, with a concommitant increase in the level of residual glucose. This was similar to the results discussed above. Although the maximum specific glucose consumption rate increased with temperature, the maximum specific growth rate did not vary much over this temperature range. More importantly, the ethanol productivity rate increased from 1.66 to 4.31 g/g/h when the temperature was raised from 30 to 43°C and it was even still relatively high at 48°C (3.98 g/g/h). The ethanol productivity rates for this organism were comparable with data obtained at low temperatures (581). A similar pattern emerged in a molasses fermentation with this strain, where ethanol yields were maximal at 40°C. However, the ethanol yields from molasses were generally lower than from a synthetic medium containing glucose (maximum yield ~83%), possibly due to the presence of non-assimilable reducing sugar complexes in the molasses (567).

The relatively high specific ethanol production rates and yields and sugar consumption rates, obtained at temperatures ≥40°C, suggest that this species may have industrial application. Mansoor *et al*. (580) concluded that *Kluyveromyces fragilis* CSIR Y326 had all the desirable properties for a successful SSF process. Among other properties, it had a high ethanol productivity rate at high temperatures (Table 16).

b. *Candida spp.*

In the Tate and Lyle study, Hacking *et al*. (579) examined 15 strains of *Candida spp.* for fermentation of glucose. Five strains gave good ethanol yields at 37°C, 4 gave good results at 40°C, and only one was capable of growing and fermenting at 45°C (Table 21). In fact this strain, *C. pseudotropicalis* YCa9 was the best performer found in this study, as it produced ~94% of the theoretical ethanol yield at 40°C. Hacking *et al*. (579) concluded that the commercial utility of *C. pseudotropicalis* would be limited because of its inability to utilize maltose which is often a major component of hydrolyzed corn mashes (582). However, a "thermophilic yeast"

C. pseudotropicalis C-23 has been used in some Cuban distilleries with good results (583, 584). Contreras *et al.* (583, 584) made a comparative study between C-23 and a traditional *S. cerevisiae* strain over a 5 month period. They concluded that the fermentation efficiency was 10.2% higher for C-23. Unfortunately, no information was provided as to the fermentation temperatures employed in these studies.

McCracken and Gong (21) examined fermentation of glucose at 40°C in 8 *Candida spp.* isolated from sugar cane compost. Strains HT1, HT4 and HT7 gave the highest ethanol yields (Table 17). Strain HT4 with a T_{max} of ~48°C was examined carefully to see if it would be suitable for either an SSF cellulase process or for one that would be used in combination with xylose isomerase to carry out isomerization and fermentation of xylose. The doubling time was ~2 hours in glucose or xylose medium, although it fermented the latter sugar poorly. At 45°C, it yielded 4.3% (w/v) ethanol in 2 days from 10% (w/v) glucose. The initial fermentation rate was relatively constant over a temperature range of 30-50°C (Figure 6). At 50°C, ~2% (w/v) ethanol was produced from 4.5% (w/v) glucose in 6 hours. It also fermented the glucose portion of neutralized and pretreated sugar cane bagasse at 45°C (21).

In an SSF process with *T. viride* cellulase, the fermentation rate of *Candida sp.* HT4 increased when the temperature was raised from 30 to 45°C (21). At 30°C and 45°C, the productivities in 48 hours were 20.8 and 31.5 g/ℓ/h, respectively. *Candida sp.* HT4 also fermented glucose at 50°C; however, at this temperature the rate was much slower, probably as a result of cell death. The results also indicated that significant quantities of ethanol were produced from xylose by *Candida sp.* HT4 at 45°C, when xylose isomerase was added to the medium. Therefore, this strain might have promise in industrial alcohol production.

In other SSF process trials investigators reported good performance of *C. acidothermophilum* ATCC 20381 (585) and *C. brassicae* IFO 1664 (586, 588, 589) at 40°C. The latter yeast especially, may have good industrial potential. From over 300 strains of yeast screened, *C. brassicae* IFO 1664 had by far the most prolific growth in spent sulfite liquor and unlike most yeasts, it was able to metabolize inhibitory substances present in the liquor (401, 402). Other *Candida spp.* capable of fermentation at temperatures ≥40°C are shown in Table 17.

c. *Saccharomyces spp.*

Of 14 strains of *Saccharomyces spp.* examined by Hacking and co-workers (579), 13 gave reasonable ethanol yields at 37°C and 3 at 40°C. It is interesting to note that 90% of *S. cerevisiae* strains examined by Cooke (570), grew at 37°C. Although none of the *Saccharomyces* strains in the Tate and Lyle study grew or fermented at 45°C, if grown at 40°C and then allowed to ferment at 45°C, some ethanol was detected (~2-3% v/v), (Hacking - personal communication).

The second and third best strains at 40°C in the Tate and Lyle study were *S. cerevisiae* YSa86 and *S. uvarum (carlsbergensis)* YSa85. Others have also reported good fermentation of *Saccharomyces* strains at this temperature (see

Tables 18 and 19). Blotkamp *et al.* (586) found high ethanol productivity rates at 40°C of *S. cerevisiae* ATCC 4132 in a SFF process with the cellulase of *T. reesei*. *S. uvarum (carlsbergensis) inulyticus var. nov.*, a new ethanol tolerant inulin-fermenting species from rice wine, produced 9.8% (v/v) ethanol from the stepwise addition of sucrose (596). Hawke *et al.* (594) reported a number of *Saccharomyces* strains (species of *S. cerevisiae*, *S. chevalier*, *S. diastaticus*, and *S. uvarum (carlsbergensis)* that were able to ferment 40% sweet sorghum juice at 41°C (as measured by CO_2 production).

Lee *et al.* (597) examined the fermentation kinetics of *S. uvarum (carlsbergensis)* in batch culture over the temperature range 25-43°C. As seen in Table 24, the ethanol yield was constant over this temperature range. The optimum temperature for growth was 33°C while the maximum rate of ethanol production was constant at 37-43°C. It was also shown that increasing concentrations of ethanol exerted a greater degree of inhibition on the growth rate than on ethanol formation (597).

Haraldson and Bjorling (598) screened 20 *Saccharomyces* strains for ethanol production at high osmotic pressure and temperature (30, 40, and 50° Brix cane molasses at 32-45°C). Many yeast strains gave good ethanol productivities at 42 and 45°C on 30° Brix molasses (~33.81% w/v), however, most of them performed poorly at this temperature on 50° Brix molasses (Table 25). *Schizosaccharomyces pombe* strains, Alfa-Laval CBS 357 and CBS 352 performed reasonably well on 50° Brix molasses, and the latter strain continued to do so in extended studies (598).

Chye and Meng (599) reported that a "rice cake yeast" produced ~11% (w/v) ethanol at 40°C after 72 hours from pineapple wastes enriched with cooked sago. From an extensive screening procedure, the By-Product Development Department of the Phillipines Sugar Institute identified a Lambanog yeast isolate which fermented a molasses mash at 40-42°C with an efficiency of ~82.5% (600). Samaniego and Olan (601) undertook a study to test the performance of this promising isolate at high temperatures. From a molasses mash containing 10% reducing sugars, reported efficiencies (average of duplicate runs) of the Lambanog isolate were 74.7% at room temperature, 76.6% at 30-32°C, 82% at 35-37°C, 80% at 40-42°C, 70.7% at 45-47°C, and 59.2% at 50-52°C (602) (Table 20). Due to the demonstrated experimental procedures and the information given in Samaniego and Olan's report (601), their results should be interpreted with caution. It appears that the reported fermentation temperatures were achieved in the flasks after the first 8 hours of incubation at 30°C. Since these researchers did not report inoculum size, ethanol values, or sugar levels after the first 8 hours of incubation, it is conceivable that some of the ethanol was formed during this part of the fermentation. The authors did however, measure the rate of fermentation by measuring the loss of weight which they claimed was due to the evolution of CO_2 and which corresponded to the rate of ethanol production. In this regard, they showed that there was some weight loss of fermentation during the first 8 hours. However, they also showed that the loss of weight varied significantly after the first 8 hours, and that there was a greater loss of weight at the lower temperatures. From their results it appears that some fermentation took place at 45-47°C and probably at 50-52°C. It is possible however that the loss of weight was due to media evaporation or masked by simultaneous biomass production.

Stewart and co-workers (595) investigated the fermentation ability of a fusion product (strain 1400) at 40°C in a 20% glucose medium (Figure 7). The two fusion partners consisted of *S. diastaticus* 1384, a diploid strain having the ability to metabolize dextrin, and *S. uvarum (carlsbergensis)* 21, an alcohol tolerant polyploid brewing strain. Although *S. diastaticus* produced 6% (w/v) ethanol in 24 hours, strain 1400 produced 7% (w/v) ethanol during this time period, and 8% (w/v) in 60 hours, with 80% of the initial glucose being metabolized (Figure 7). Strain 1400 has recently been investigated for its ability to ferment corn and wheat mashes for industrial ethanol production with reduced concentrations of added glucoamylase. Ethanol yields and fermentation efficiencies were unaltered at up to a 50% reduction in saccharifying enzymes, thus offering significant cost savings (Whitney - personal communication). This reduction in enzyme concentration was possible because strain 1400 is known to contain the dextrin (*DEX*) genes of *S. diastaticus* which enable it to produce and secrete its own glucoamylase (595, 602).

Seki and co-workers (591) recently examined in batch fermentation, fusion products of two strains of *S. cerevisiae*, N1 and TJ1. *S. cerevisiae* N1, isolated from an Awamori brewing mash, produced 9.6% (w/v) ethanol at 40°C in 35% (w/v) glucose medium, while the other parent TJ1, isolated in Thailand, produced ~7% (w/v) ethanol under the same conditions. One fusion product, strain AM12, produced 12.4% (w/v) ethanol at 40°C (Figure 8). This fusant produced 16.3, 14.0, 12.4, and 8.4% (w/v) ethanol at 30, 35, 40, and 42°C, respectively. Although the production rate was highest at 35°C, the yield of ethanol based on glucose consumed was ~90% of the theoretical at all four temperatures. In addition, strain AM 12 grew faster than the parents at 30-42°C, and ethanol production and flocculence remained stable at pH 3.5-5.5. Seki *et al.* (591) also tested strain AM 12 in batch culture with cell recycle at 37°C. The cells were cultivated in 3 ℓ fermentors containing 1.5 ℓ of 20% (w/v) glucose medium. Upon producing 10% (w/v) ethanol, cells were allowed to sediment and 1.2 ℓ of the upper portion of the broth was withdrawn and replaced with fresh medium. This recycling procedure was repeated 10 successive times, and each time ~8% (w/v) new ethanol (10% in total) was produced in 6 hour cycles. The ethanol productivity at 37°C was 13.3 g/ℓ/h. It would have been interesting to study how this strain behaved under recycling conditions at temperatures ≥40°C, but the authors made no mention of attempting this.

Takagi and co-workers (603) reported that homozygous polyploid strains fermented similarly to parental haploid strains. They also noted a significant increase in the fermentation rate of glucose by heterozygous hybrids constructed from two independent strains. Therefore, the high production of ethanol by hybrid fusants appears to be due to "consolidation in the pathway of ethanol production by complementation", and not simply due to the physical increment of cellular volume by higher ploidy (603). In this regard, it is possible that spheroplast fusion (reviewed in 604) may be a means of generating ethanol tolerant and/or thermotolerant strains of yeasts. Although other fusion products of various species of *Saccharomyces*, *Schwanniomyces*, *Kluyveromyces*, and *Candida* exist (605-611), unfortunately they have not been examined for their fermentation ability at high temperatures.

A strain of *Saccharomyces ellipsoideus*, originally isolated from pineapple in Guatemala, was adapted to 20% (v/v) ethanol (Castro-Conde - personal communication). This strain produced 16% (v/v) ethanol from molasses at 39°C in less than 48 hours in industrial fermentations carried out in 48,000 gallon fermentors, employing a four step incremental system of fermentation. It was found that this strain produced a proteolytic enzyme bvromeline), as well as some zymase and phosphatase. The high yields of ethanol were attributed to the ability of the proteolytic enzymes to hydrolyze proteins found in molasses, which may have formed a protective colloidal barrier to the fermentation process (Castro-Conde - personal communication). Perhaps, the high temperature of fermentation resulted in some lysis which enabled the proteolytic enzymes to be released from the cell.

Hacking *et al.* (579) concluded from their study that yeast strains with adequate performance at 40°C can be isolated. They also concluded that the large differences in the growth and fermentation temperature optima would make thermophilic yeast fermentation processes impractical for industrial application (579). However, it is conceivable that the differences in temperature optima between growth and fermentation may even be beneficial for a thermotolerant yeast fermentation adiabatic system process, especially in the tropics. A 2-step fermentation process could be visualized where initially a yeast would grow rapidly at ambient temperature (~20-30°C) and its growth rate would increase with time due to the exothermic fermentation process. The gradual rise in the temperature of the fermentation medium would eventually slow down growth, and enhance the fermentation rate. The strategy would be to ensure that there would be many cells present before the high temperature was reached. In this regard, recycling procedures might work well for thermotolerant or thermophilic fermentation processes. This two stage fermentation process also has potential for employment of temperature-sensitive (ts) yeast mutants for industrial processes (612-614). This has been extensively reviewed by Slapack (615). Initial cultivation of a ts mutant at lower permissive temperatures would favor rapid growth. The subsequent rise in temperature due to the exothermic fermentation reaction would favour expression of the desired mutant phenotype such as increased methionine content for SCP production (615).

VII. ETHANOL PRODUCTION FROM CELLULOSIC BIOMASS

Cellulose, a high molecular polymer of glucose units linked in β-1,4 configuration, is an orderly crystalline structure associated in a matrix with hemicellulose and lignin (616). Lignocellulose feedstocks contain different proportions of these components which vary with the source (380). Typical ranges are: 35-50% cellulose, 20-30% hemicellulose, and 20-25% lignin (341). In this review the term "cellulosics" refers to only the cellulose components, while the term "hemicellulose" refers to the hemicellulose fraction - mostly a pentose polymer of various structures (eg., xylan) and in some cases hexose polymers such as mannan. Biomass is usually defined as all plant and animal material which can be converted into energy.

Cellulose, the world's most abundant renewable resource is available in the form of domestic, agricultural, and industrial wastes. Since its utilization alleviates some pollution problems and it is not required as a foodstuff, it is a logical raw material with which to base future chemical needs. Significant advances have been made over the last decade in unique approaches to cellulose conversion, as evidenced by a number of recent reviews (377, 381, 617-624). In fact, there is a global research effort underway to evaluate the use of cellulosics for the production of ethanol and other chemical feedstocks (341, 383, 625-634). Alcohol from cellulose is not a new concept as converting cellulose to sugar for making alcohol has been practiced for many years.

Although fermentations of cellulosics produce low ethanol yields, other novel fermentation processes for utilizing biomass have been recently proposed. For example, biorefining of biomass to fuels and chemicals, the overall process of suppressed methane fermentation to organic acids followed by electrolysis (635). Armstrong *et al.* (636, 637) recently demonstrated the feasibility of using *Candida utilis* for production of ethyl acetate or acetaldehyde from dilute ethanol streams. Both these compounds are of higher economic value than ethanol. Acetaldehyde, with a low boiling point of 21°C, readily evaporates, and can therefore be easily recovered from the fermentation broth (636). This process, has recently been filed for patenting (Armstrong - personal communication). Although it most probably will not be practical for cellulose fermentation due to the low ethanol concentrations needed, it may have other industrial applications.

There are essentially six major routes for the production of ethanol from cellulosic biomass (see Figure 9). The conventional two step approach consists of either enzymatic or chemical hydrolysis of cellulose to glucose. This is followed by the conversion of glucose to ethanol, usually with yeast or *Zymomonas spp.*. There are a number of current reviews available on both acid hydrolysis (370, 638-640) and enzyme hydrolysis (616, 641-646). Whereas acid hydrolysis is much faster than enzymatic treatment, the degradation products from the former lead to lower yields of sugars, production of inhibitors, corrosion of equipment, and high capital costs. Enzymatic hydrolysis, which usually employs the cellulases from *T. reesei* is also an expensive process. The saccharification of cellulose represents up to 60% of the total cost of producing ethanol from cellulose (642). Cellulose is highly resistant to microbial or enzymatic attack. Therefore, even for the other ethanol from biomass processes (i.e., mixed culture fermentation, SSF, etc.), an additional costly pretreatment of the lignocellulosic material is often essential (435, 625).

Two stage processes that employ two different organisms avoid the need of costly enzyme for saccharification. However, as the name suggests, the fermentation takes place in two stages, and is therefore still expensive. Simultaneous saccharification fermentations (SSF) require the enzymatic saccharification and simultaneous fermentation to take place in the same vessel. The use of thermophilic enzymes and/or thermotolerant yeasts in this process could make it even more attractive if more ethanol tolerant strains could be developed. *C. thermocellum* monoculture fermentations directly convert cellulose to ethanol, thereby eliminating the need for expensive enzyme or acid treatment.

If cellulose is to be economically converted to ethanol, the hemicellulose fraction containing the pentoses must also be effectively fermented. As already pointed out, despite various efforts, no yeast or *Zymomonas* species can efficiently ferment the hemicellulose fraction of biomass, nor can they metabolize cellulose. An alternative process is the single step conversion of cellulose to ethanol. In this process, cellulose is metabolized to ethanol by a defined mixed culture consisting of *C. thermocellum* and either *C. thermohydrosulfuricum*, *C. thermosaccharolyticum*, or *T. ethanolicus*. This takes advantage of the cellulolytic and hexose fermenting ability of *C. thermocellum*, and the hemicellulolytic and hexose and pentose fermenting ability of the glycolytic ethanologens. It should also be noted that Kahn and co-workers at the NRC, Canada, have conducted studies on mesophilic mono- and co-culture fermentations of cellulose (647-652). Although they will not be discussed in this review, it will suffice to say that they have encountered similar problems to the thermophilic bacteria (i.e., low ethanol tolerance).

A number of programs, many sponsored by the Department of Energy, have resulted in development of different processes to convert cellulose to ethanol, (mixed culture fermentation, SSF, etc.). A number of them involving thermophilic bacteria or thermotolerant yeasts will be discussed in subsequent sections. It becomes apparent that the cellulolytic ethanologenic bacterium *C. thermocellum*, is fundamental to the direct conversion of cellulosic biomass to ethanol at high temperature. Therefore in the following section its cellulase system and means of improvement will be reviewed in depth.

1. Characterization of the Cellulase System of *C. thermocellum*

The term cellulase is generally used to designate a complex of enzymes which act synergistically to hydrolyze cellulose to its simple sugars (653-655). The cellulase systems of *Trichoderma reesei* and many other fungi have been extensively studied (653, 656-658). In general, there are four principle enzymes of the fungal cellulase system. Endo-1,4-ß-glucosidase hydrolyzes the cellulose chain randomly forming short chains of oligosaccharides. The 2 exoenzymes, exo-1,4-ß-glucosidase and exocellobiohydrolase then split off glucose and cellobiose units, respectively, from the non-reducing end of cellulose or the short chain oligosaccharides. Finally, a ß-glucosidase (cellobiase) hydrolyzes cellobiose. Similar enzyme systems exist for hemicellulose (659).

Several thermophilic microorganisms can degrade cellulose. *Actinomyces*, *Sporocytophaga*, fungi, and *Clostridia* (526, 528, 660-666). Recently, Margaritis (80) published a comprehensive review on the cellulases of thermophilic microorganisms. There are many disadvantages to the fungi; they may produce toxic substances, they grow slowly, and ethanol if it is produced, is present only in minute quantities. *C. thermocellum* is the only thermophilic cellulolytic microorganism capable of producing ethanol as a major fermentation product, however this amount is still very low (≤0.5% w/v).

The general properties of *C. thermocellum* have been reviewed in a preceeding section (Section V, 2c). *C. thermocellum* is the only bacterium shown to date to produce a true cellulase system (503). Using anaerobic bacteria to achieve cellulose hydrolysis has many general advantages, eg., avoidance of mycelia formation reducing mass transfer problems (667). The cellulase system of *C. thermocellum* is beginning to receive attention as indicated by the isolation of a number of intermediate enzymatic activities (515, 668).

The purification and characterization of the cellulase system of *C. thermocellum* has been plagued by the following factors (503, 669): use of different strains of *C. thermocellum*, low amounts of extracellular protein, adherence of enzyme to cellulose, tendency of proteins to form aggregates, and low recovery of the components during purification.

The crude cellulase of *C. thermocellum* is similar to *T. reesei* in having a multicomponent nature, being inhibited by cellulose hydrolysis end products, and degrading Avicel microcrystalline cellulose or hemicellulose (503, 515). However, the cellulolytic apparatus between the two organisms differs considerably as follows (503, 512, 515, 670-673): *C. thermocellum* has a higher endoglucanase/exoglucanase activity ratio, and lacks extracellular cellobiase, β-xylosidase, and proteolytic activity. It produces more long-chain oligosaccharides instead of short ones as initial (15 min) hydrolytic products; final products are mainly cellobiose or xylobiose. *C. thermocellum* grows faster on cellulose or cellobiose and its cellulase retains higher specific activity and stability at 60-70°C. The two organisms also differ in their cellulases in response to different enzyme inhibitors and minerals.

C. thermocellum strains produce an extracellular cellulase complex which solubilizes carboxymethylcellulose, microcrystalline cellulose or xylan (512, 515, 516, 668, 672). Zeikus *et al.* (27) compared the cellulolytic enzyme system of *C. thermocellum* LQR1 with a hypercellulase mutant *T. reesei* QM 9414. Under reaction conditions optimized for each enzyme, the fungal cellulase was considerably more active than the thermophile. However, as discussed in Section VII, 1d, i, under certain conditions, the cellulase system of *C. thermocellum* has a much higher specific activity on crystalline cellulose than the fungal cellulases.

Ait *et al.* (668) partially purified an enzyme complex from *C. thermocellum* grown on cellulose and postulated this to be an endoglucanase.
Endo-β,1,4-glucanase was purified to apparent homogeneity for 2 strains of *C. thermocellum* by two independent groups of workers (674, 675). The enzymatic

properties (i.e., molecular weight, pH, and temperature optima) reported by the 2 groups were very different and it was concluded that 2 distinct endoglucanases were isolated (441). Closer observation showed that the enzymes isolated from Petre's (675) and Zeikus' group (674) were from cells grown on cellulose, and cellobiose, respectively. However, it is apparent that several endoglucanases and exoglucanases may be produced by *C. thermocellum* (441, 669). A further endoglucanase and exoglucanase have also been isolated and characterized from other thermophilic *Clostridium spp.* (536, 538). The extracellular nature (95% extracellular) of the endo-ß-glucanase was observed (516). This finding indicated that the 35% figure obtained in a cellulose fermentation by Gordon *et al.* (676), was mainly due to cellulase adsorption to insoluble cellulose, rather than to it being intracellularly located.

Sih and McBee (677) found that cellobiose phosphorylase was present in cell free extracts of *C. thermocellum*. Sheth and Alexander (678-681) have isolated and studied a cellodextrin phosphorylase and a cellobiose phosphorylase from *C. thermocellum*. This coupled with the apparent lack of extracellular cellobiase has led to speculation that cellodextrins and cellobiose are transported into the cells and then are phosphorolytically cleaved to form glucose and glucose 1-phosphate (G1P) through the action of these intracellular enzymes. The conversion to G1P over glucose benefits *C. thermocellum*; the phosphorylated glucose is more easily metabolized, does not inhibit the fermentation, and also preserves the energy of the glucosidic bond (441, 680).

Although *C. thermocellum* lacks a true extracellular cellobiase, a cell-bound ß-glucosidase which appears to be constitutive has been purified to apparent homogeneity (682). The purified enzyme was specific for ß configurations and was found to be active on ß-glucosides and cellobiose (682-684). The localization of this enzyme at the cell surface could allow for the hydrolysis of cellodextrins and cellobiose. Although, it is not yet obvious that ß-glucosidase is involved in cellulolysis (684) it may be that cellodextrins and cellobiose can be metabolized by either of two mechanisms: hydrolysis by ß-glucosidase, or phosphorylation by intracellular cellodextrin and cellobiose phosphorylase (Figure 10). This ß-glucosidase is very heat stable and may be of significant interest in the industrial saccharification of cellulose (683).

a. Cellulosome or Cellulose-Cellulase Complex

Su *et al.* (685) found that despite vigorous stirring, *C. thermocellum* cells were bound to the cellulose substrate in the initial stages of fermentation. However, other results showed that contact between *C. thermocellum* cells and cellulose was not necessary for effective depolymerization (503, 686). Further studies by Lamed *et al.* (687, 688), showed that adherence of cells of *C. thermocellum* to cellulose was mediated by an antigenically active cellulose binding factor (CBF) located on the cell surface. The observed adherence was specific for cellulose and was not significantly affected by parameters such as salts, pH, temperature, detergents, and soluble sugars. They proposed the term "cellulosome" to describe the highly organized multicellular structure which they thought was responsible for both cell adherence as well as the synergistic interaction between the specified cellulolytic components (689). Results suggest that previous information reported for the crude cellulase apparatus of *C. thermocellum* essentially reflects the contribution of the cellulosome (690). The cellulosome exhibits similar true cellulase activity, specific activity and rate of hydrolysis and similar responses to calcium, thiol, cellobiose, and low ionic strength (690).

Wiegel and Dykstra (691) found that attachment of *C. thermocellum* to cellulosic fibers occurred via a fibrous ruthenium-red staining material. Although it has not been fully characterized, they concluded that the attachment is involved in cellulose metabolism. They postulated that under conditions unsuitable for growth, *C. thermocellum* gains an advantage by sporulating while remaining fixed to its insoluble substrates.

It has been known for many years that cellulose degradation by *C. thermocellum* is accompanied by the production of a water insoluble "yellow substance" (YS). The YS is attached to the cellulose fibers and effectively binds endoglucanase to the cellulose fibers (692). Ljungdahl *et al.* (692) postulated that the YS also aids in adhesion of the cells to the cellulose fibers and may even be directly involved in the hydrolytic process. However, other results suggest that the YS may have an alternative role unrelated to affinity (690). It is also possible that the fibrous red staining material described by Wiegel and Dykstra (691) is the same YS (692). Purification of the YS may lead to a better understanding of the importance of the cellulosome.

b. Problems of Measuring Cellulase Activity

A fair comparison of cellulase activity cannot really be made due to variations in methodology between laboratories, and the heterogeneity of the cellulosic material employed as the substrate. For example, the yield of cellulase may depend on the time and temperature of pretreatment (619). Cellulase production is complicated by the fact that different types of enzyme activity are required for cellulose hydrolysis. Furthermore, these enzymes are not single proteins, and it is not clear how many proteins are involved. Despite the extensive body of knowledge on the properties of *C. thermocellum* cellulases, the mechanism of hydrolysis is not yet completely understood. Since cellulose absorbs strongly to cellulase this causes a decrease in measured enzyme activity. It is not known if this absorption is equivalent for the different enzymes in the cellulase complex (503, 619).

The extent of hydrolysis of various cellulosic substrates is believed to be a far more realistic assessment of the potency of a cellulase preparation of *C. thermocellum* than are units of specific activity (686). The latter measurement is only useful when the preparation has been shown to contain true cellulase activity. According to this, the majority of results which measure cellulase activity of *C. thermocellum* after short incubation periods, have little meaning.

The majority of studies use commercially available purified cellulose substrates such as solka floc; a wood cellulose that contains both cellulose and hemicellulose. However, there are even many different types of cellulose in solka floc. Since solka floc is highly unlikely to be used as a substrate in any industrial process, studies should be conducted on more pertinent substrates such as pretreated wood. Lignocellulosics found in nature represent complex ill-defined systems in which a variety of secondary effects may be observed. For example, lignocellulosics with a high degree of tannins and phenols may inhibit cellulase activity (435). In this regard, many researchers (439, 693-695) have shown that systems which operate on pure cellulose can be greatly inhibited by more practical substrates such as corn stover or steam exploded wood. Far too few laboratories have reported testing multiple substrates.

It becomes evident that the choice of cellulose substrate is important for cellulose preparations. Cotton appears to be a suitable substrate for cellulose preparation because its fibers are representative of the most resistant cellulose fraction found in natural polysaccharides (686).

c. Regulation of Cellulase Activity

The growth rate of *C. thermocellum* on a soluble substrate such as cellobiose is faster than the growth rate on an insoluble substrate such as cellulose (515, 696). This indicates that the solubilization of cellulose is a rate-limiting step for growth. However, specific rates for ethanol production can be higher on insoluble substrates than on soluble ones (667). The uptake of hydrolytic products (soluble sugars) is also a rate-limiting step in *C. thermocellum's* metabolism (667, 696).

In order to improve the cellulase activity of *C. thermocellum*, its regulatory mechanisms of enzyme synthesis must be better understood. Cellulase synthesis has been reported to be inducible by cellulose in several fungi and bacteria (503). A model has recently been proposed by Gong and Tsao (697) for the regulation of cellulase biosynthesis in fungi. The regulation of bacterial cellulase production has not been examined in detail, but appears to be controlled by catabolite repression and induction (503).

There is much disagreement in the literature on the constitutive nature of the cellulase system of *C. thermocellum*. Various studies conclude that the cellulase system is constitutive and not subject to catabolite repression (512, 516, 518, 519, 672, 675, 698, 699). However, a number of other papers conclude that the main cellulose hydrolytic products (cellobiose and glucose), inhibit the production of cellulase i.e., the cellulase system is subject to catabolite repression (141, 450, 515, 686, 700). Similarly, some strains (eg., *C. thermocellum* LQR1) induce the cellulase system only with cellobiose as a substrate, while others (eg., *C. thermocellum* LQ8) produce cellulase only in the presence of cellulose (515).

The existence of β-glucosidase has also been the subject of debate (453, 515, 519, 698). The fact that cellobiose strongly and glucose weakly inhibited cellulase activity sometimes, may be dependent on the nature of the substrate and nutrients, and due to strain differences. It may be due to the low uptake of glucose at low yeast extract concentrations used in some studies. Some researchers (700) found that β-glucosidase added to the medium lessened the inhibitory effect of cellobiose on the cellulase system. Therefore, it would be very useful to develop strains that secrete cellobiase.

Much lower quantities of cellulase are obtained when sorbitol or fructose is the carbon source (503). The mechanism by which these carbon sources prevent cellulase synthesis is not known, but some control might be exerted by their transport mechanism (503).

Various strategies are available in achieving higher cellulase yields: optimizing cultural conditions, optimizing enzyme secretion by addition of compounds that make the cell wall more permeable, and by genetic manipulation. These will be covered in the next sections.

d. Effect of Physiological Factors on Cellulase Synthesis

i. Nutritional Manipulation

Two independent studies carried out to determine optimal initial cellobiose levels for growth and cellulase production have given similar results (516, 701). Maximal growth and enzyme production were reached between 0.5 and 2% (w/v) cellobiose. Although 0.2% (w/v) cellobiose supported growth, there was none evident on 5% (w/v) cellobiose. Mcbee (502) concluded that complex nitrogenous sources do not stimulate cellulase production. However, Garcia-Martinez *et al.* (516) showed that the type of nitrogen source could influence the cellulase yield. They observed optimal cellulase activity with 0.2% (w/v) urea as a nitrogen source.

Many chemically defined media without yeast extract have been developed for *C. thermocellum* (502, 670, 702, 703). Required growth factors were vitamin B_{12}, biotin, pyridoxine, and p-aminobenzoic acid (703). Saddler and Chan

(428) recently reported that DSM media originally described in the catalogue of the Deutsche Sammlung von Microorganism for the growth of *C. thermocellum* was superior to other media tested.

Enebo (141) found that casein hydrolysates enhanced cellulase production. Duong *et al.* (503) reported increased ethanol production (30-50%) by a number of crude fermentation additives (corn steep liquor, beef extract, pancreatic or tryptic casein digests, and blood hydrolysates). Unlike fungi, the addition of surfactants such as Tween 80 or 20 did not increase cellulase production in *C. thermocellum* (503).

The cellulase system of *C. thermocellum* has been reported to be insensitive to oxygen (515, 675). However, it was recently shown that it was stimulated by thiol-reducing agents such as dithiothreitol (DTT) (686) and is oxidatively inactivated by air (704). Ng and Zeikus (672) found that the cellulase system of *C. thermocellum* is inhibited by Hg^{+2}, Cu^{+2}, and Zn^{+2}, while Ca^{+2} and Mg^{+2} had no effect. The basis for this inhibition is unknown (503).

Recent data by Johnson and Demain (704) suggest that the cellobiohydrolase component of the cellulase system contains essential sulfhydryl groups. In addition, since this component is stimulated by iron, calcium, or thiol, they postulated that it contained a transition metal that participated in cellulose depolymerization (686, 690, 704, 705). It was recently observed that upon inclusion of a thiol-reducing agent and calcium, the cellulase system of *C. thermocellum* has a much higher specific activity on crystalline cellulose, as compared to the fungal cellulase systems (704, Afeyan - personal communication). However, to date productivity (amount of protein per biomass) is still low as compared to hyperproducing cellulase mutants of *T. reesei*.

ii. Temperature and pH

The optimum pH and temperature for cellulase activity are different from those for culture growth of *C. thermocellum* (706). Therefore, cellulase activity might be improved by changing these parameters during the stationary phase of the culture. Optimum temperature for cellulase production varies between strains; some have reported 55-60°C (505, 707) while others have found maximal activity at 70°C (686). It may be possible to further enhance the thermal stability of cellulase by employing the techniques of immobilization (145) and intramolecular crosslinking (80, 503).

Similarly, differences exist in pH optima between strains. For example, Mcbce (502) found the optimum pH for cellulose decomposition varies from 6.4 to 7.4, whereas, in a different strain Johnson *et al.* (686) found maximal activity at pH 5.7 to 6.1. Another study has shown optimal activity at pH 8.9 (708). The optimum pH of xylanase activity in *C. thermocellum* was found to be 6.5-7 (709). Garcia-Martinez *et al.* (516) found that retarding the pH drop in tubes or flasks which occurs with acid formation improves growth and cellulase titre. The effect of pH control of *C. thermocellum* growing on cellulose in fermentors has been recently examined (676, 710). Without pH control, the pH value dropped to 5.5 and growth stopped. Maintaining pH at ~6.3 or 7, increased biomass, cellulose degradation, utilization of sugars formed, and production of ethanol and acetic acid. In a continuous culture of *C. thermocellum* on cellulose, to control pH, sodium bicarbonate buffer had the most preferred effects on growth rate and ethanol production (696, 711).

iii. Agitation

C. thermocellum strain JW20 degrades cellulose faster under agitation than without it in both mono- and co-culture (712). However, Wang *et al.* (699) found that high agitation (200 rpm) was detrimental to their *C. thermocellum* culture and recommended 100 rpm for future use. Furthermore, ethanol production ceased earlier in agitated continuous cultures of *C. thermocellum* grown on cellulose (696, 711).

Su *et al.* (685) examined the effect of stirring on *C. thermocellum* growth and product formation. It was observed that stirring decreased the ethanol/acetate ratio 3 fold; both by decreasing ethanol and increasing acetate levels. Their results demonstrated that the H_2 concentration in the medium played an important role in regulating product formation. The higher ethanol/acetate ratio obtained in unstirred cultures was due to the accumulation of H_2 in the medium which inhibits acetic acid formation (Section IX, 1b, ii). In fact, addition of hydrogen to the gas phase of *C. thermocellum* YS increased the relative ethanol productivity (Figure 11) (685, 713). However, the addition of H_2 had little effect on *C. thermocellum* LQR1 (461). Stirring of the culture facilitated H_2 removal and relieved the inhibition of acetate production. However, at high H_2 pressures, the ethanol/acetate ratio remained relatively constant with increased rates of stirring (Figure 11). These results suggest that improvements in *C. thermocellum* anaerobic fermentation processes might be achieved with proper fermentor designs for maximizing the H_2 concentration. In this regard, the vacuferm process might be unsuitable for this organism while Mulholland's CO_2 stripping process may be ideal (Section IX, 4a).

iv. Immobilization

An alternative approach for producing cellulases is to use immobilized whole cells, which on releasing cellulase, are easily separated from the cellulase system (146). Carrageenan gels and bituminous coal particles have been used to immobilize *C. thermocellum* cells (705, 714). The former system increased the productivity of the cellulase complex (705). Efforts are presently underway at MIT to study *C. thermocellum* in co-culture with *C. thermosaccharolyticum* in a "special immobilized cell packed bed configuration" (Afeyan - personal communication).

e. Genetic Manipulation of Cellulase Production

Cellulases obtained from fungal mutants are generally considered the most active cellulases. However, most of the screening processes for hypercellulase mutants have been carried out with *T. reesei* (715-719). Very few attempts have been made to genetically improve cellulase production of *C. thermocellum*. Strain improvement has been impeded because little is known about the genetics of this bacterium. In addition, the limited biochemical characterization of the cellulase system and the extracellular nature of the cellulase complex has made it difficult to develop selective conditions for isolation of hyperproducing mutants (503).

Cellulolytic and/or hemicellulolytic mutants possessing the following characteristics should be developed (718-720). They should constitutively express cellulase activity, and produce high cellulase yields and minimal amounts of other proteins. Furthermore, they should not be subject to end product inhibition or catabolite repression.

Gomez and co-workers (721, 722) undertook a research program to develop the genetic principles in *C. thermocellum*. They found that this *Clostridium* was susceptible to mutagenesis by UV light and gamma radiation, but was resistant to nitrosoguanadine (NTG). They extended this work to the isolation of nutritional auxotrophs (722) which should allow further studies on its recombination system.

Shinmyo *et al.* (512) isolated a mutant (AS-39) which produced twice as much CMCase and Avicellase as the wild type; however, this mutant was unstable (503). Recently, mutant strains of *C. thermocellum* have been obtained which displayed higher cellulase activities than their parents (503). However, efforts in this direction have only just begun. Leatherwood and co-workers (723) have recently initiated a study to select and subsequently mutate thermophilic bacteria to increase cellulose utilization.

The problem with the isolation of cellulase overproducers has been the development of quick screening methods that will allow rapid detection. Some non-selective techniques have been used to isolate improved cellulase degrading strains, eg., rapid serial transfer in cellulose-containing media and subsequent screening for colonies with large clearing zones (503, 705, 713, 718). Tang *et al.* (709) recently developed a trinitrophenyl-xylan assay which can be used to detect xylanase secreting cells (i.e., overproducers) on agar plates. It has been shown that some non-metabolizable cellobiose analogs (salicin, arbutin, and lactose) inhibit cellulase (700). It might be possible to select for a cellulase hyperproducing strain of *C. thermocellum* by including one of these inhibitors in the medium (503). This might select for mutants which are less sensitive to cellobiose inhibition. In this regard, hypercellulase producing mutants of *T. reesei* have been isolated by the use of the glucose analog, 2-deoxyglucose, an antimetabolite and catabolite repressor (718, 719).

Developments in protoplast fusion and genetic engineering may eventually increase cellulase activity of *C. thermocellum*. Protoplast fusion of *T. reesei* has recently resulted in an increase in cellulase activity (724). Scientists at Cornell University engineered *E. coli* to digest cellulose by implanting a gene from *Thermonospora* XY that codes for cellulase production and functions at 65°C (725).

Various studies have also been undertaken to clone cellulase genes from *C. thermocellum* into *E. coli* (713, 721, 726, 727). A fragment of *C. thermocellum* DNA able to mutate *E. coli* was isolated from a gene bank developed by Gomez and Snedecor (721). Evidence indicates that this piece of DNA is an insertion sequence (721). The screening of another gene bank has led to the isolation of two genes (*cel* A and *cel* B) from *C. thermocellum* which express endoglucanase activity in *E. coli* (726-728).

2. Thermophilic Mixed Culture Fermentations

a. General Features

For years man has made use of mixed cultures (eg., lactic acid bacteria) for traditional processes such as yogurt and cheese. However, the deliberate use of defined mixed cultures in industry has only recently been seriously considered (729). The "Symba Process" for single cell protein production from potato waste was one of the few defined mixed culture systems to have been used on an industrial scale (730); however, it has recently been discontinued (731). Mixed culture systems have also proved useful for methane production from waste and manure (Table 6).

It was previously mentioned that *C. thermocellum* is capable of breaking down cellulose and hemicellulose to glucose, cellobiose, and xylose. Although ethanol is produced, the yields are low. This is in part due to its inability to use xylose, and the ability of cellobiose and/or glucose to inhibit cellulase activity. If the fermentation by *C. thermocellum* is coupled with a secondary bacterium which can ferment cellobiose, glucose, and xylose, the steady state concentration of the hydrolysis products can be kept low. As a result of the removal of these inhibitory sugars, the fermentation of cellulose can proceed rapidly. In addition to higher ethanol yields, the simultaneous occurrence of saccharification and fermentation steps in a single fermentor result in a significant reduction in capital costs.

The fermentation of cellulose with a defined mixed culture of *C. thermocellum* and an ethanologenic bacterium appears to occur in different stages as described by Carreira and Ljungdahl (441). The first stage consists of induction of the cellulase system of *C. thermocellum* and hydrolysis of the cellulose. In the second stage, hydrolysis products are removed rapidly by the ethanologenic bacterium, and both the cellulolytic and ethanologenic bacteria are active. In the third stage, the metabolic activity of the cellulolytic bacterium is inhibited by the build up of fermentation products, but the excreted cellulase system is still active. During this stage, the fermentation products are primarily due to the activity of the dominant ethanologenic bacterium Finally, the secondary bacterium is also inhibited by the fermentation products and the fermentation comes to a stop.

There are essentially two major problems preventing increased ethanol productivities from mixed culture cellulose fermentations. The first is the ethanol toxicity problem; however, mutants have been obtained which are more ethanol tolerant (Section IX, 2a). The second is that the saccharification step is the overall rate limiting step (731).

Most of the thermophilic mixed culture fermentation work with *C. thermocellum* has been conducted in 3 laboratories in the United States: 1) Wang's group at the Massachusetts Institute of Technology (MIT), 2) Zeikus' group, formerly at the University of Wisconsin and presently at Michigan State University, and 3) Ljungdahl's group at the University of Georgia. They have focused their efforts with *C. thermosaccharolyticum*, *C. thermohydrosulfuricum*, and *T. ethanolicus*, respectively. In addition, research is still continuing with these organisms at other places, eg., Forintek (695), Texaco Research Centre (732).

b. *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*

Zeikus and co-workers at the University of Wisconsin have focused most of their research efforts with these two organisms (reviewed in 27, 48, 139, 438, 733). Their use in mixed culture fermentations is now protected by a patent (734). *C. thermohydrosulfuricum* grows successfully in a stable co-culture with *C. thermocellum*. The fermentation kinetics of product formation of mono- and co-cultures of these two *Clostridia*, on 1% (w/v) solka floc is demonstrated in Figure 12. The co-culture significantly increased the amount of cellulose degradation and ethanol yield and productivity when compared to the mono-culture (27). Most importantly, the hemicellulose portion of solka floc which was not utilized by either culture alone, was metabolized by the mixed culture (456). Essentially similar higher yields and productivities were obtained in *C. thermohydrosulfuricum* mono-culture fermentations that were supplemented with the cellulase from *C. thermocellum*.

Although the metabolic basis for this enhanced ethanol production in co-culture is not completely understood, several physiological properties of these organisms help explain this phenomena (27, 139, 456, 735). They are as follows: the ability of *C. thermocellum* to degrade glucans and xylans; the ability of *C. thermohydrosulfuricum* to ferment xylose; the ability of *C. thermohydrosulfuricum* to compete with *C. thermocellum* for both glucose and cellobiose; less susceptibility of *C. thermohydrosulfuricum* to inhibition by lactate and acetate; and a lower proton concentration associated with fermentation of equivalent amounts of cellulose.

Unlike *C. thermohydrosulfuricum* and almost all other microorganisms, *C. thermocellum* metabolizes cellobiose in preference to glucose, the general catabolite repressor (735). This is because cellobiose metabolism in *C. thermocellum* is constitutive while its glucose metabolism requires induction of glucose permease. In contrast, *C. thermohydrosulfuricum* metabolizes glucose in preference to cellobiose. This is because its glucose metabolism is constitutive and its cellobiose metabolism requires induction of cellobiose permease (735). Nevertheless, *C. thermohydrosulfuricum* can successfully compete with *C. thermocellum* for both glucose and cellobiose formed during cellulose fermentation.

Ng *et al.* (456) compared mono- and co-culture batch fermentations of various cellulosics by these two *Clostridia* (see Table 26). As expected, untreated wood fermented poorly, while the SO_2-treated or steam-exploded wood fermented reasonably well. This is in contrast to results obtained by other workers (428, 695). While the ethanol/acetate ratio from the treated fermentable cellulosics ranged from ~1-1.4 in the mono-cultures, it varied between 4.9-21.1 in the co-cultures. This was determined to result from both significant increases in ethanol and decreases in acetate yields in the co-cultures. However, the final ethanol concentrations were all less than 1% (w/v).

c. *Clostridium thermocellum* and *Clostridium thermosaccharolyticum*

Wang and co-workers at MIT have conducted research with these two *Clostridium spp.* Most of their efforts have been previously reviewed (434, 439, 442, 693, 699, 736). As mentioned earlier, *C. thermosaccharolyticum* produces ethanol only in its resting state which can be obtained under conditions of limited substrate availability. In co-culture with *C. thermocellum* these conditions exist, so ethanol yields should theoretically be high. However, due to poor ethanol tolerance, ≤1% (w/v) ethanol is obtained and acetate and lactate are produced as by-products. Results have shown that through the mutation, selection and adaptation schemes, it is possible to obtain higher ethanol yields and it remains to be determined where the practical limits are.

The MIT process employs a mixed culture of *C. thermocellum* and *C. thermosaccharolyticum* that hydrolyzes the cellulose and hemicellulose components and then ferments them to ethanol. Processes were designed to handle 200 or 1500 tons per day of corn stover (737, 738). Although the energy input was only 1/3 of the energy output, the process is still at the research stage. In view of the low ethanol tolerance/productivities, there is still much research to be done before the process economics can be justified.

C. thermocellum ATCC 27405 [tolerant to ~1% (w/v) ethanol] was adapted by serial transfer to increasing concentrations of ethanol (439, 442). As a result mutants S-4 and S-6 were obtained [tolerant to ~5% (w/v) ethanol] (Figure 13) which produced higher ethanol/acetate ratios. However, they also accumulated large quantities of lactic acid, thus reducing overall yields. Wang and Fang (693) selected for LDH negative mutants and obtained *C. thermocellum* strain S-7. Although, this strain was improved (Figure 14), low yields of ethanol were obtained since *C. thermocellum* S-7 could not utilize the hemicellulose portion. Wang and co-workers (439, 693) employed a similar strategy to improve the ethanol tolerance of *C. thermosaccharolyticum* HG-2. Mutant HG-3 was obtained by adapting HG-2 to increasing amounts of ethanol; mutant HG-4 was obtained by mutation of HG-3 and selecting for low acid producers; mutant HG-6 was obtained by adapting HG-4 to progressively increasing concentrations of ethanol (Figure 15). A comparison of the fermentation yields of mutants HG-3, HG-4 and HG-6 are summarized in Table 27. It can be seen that through mutation, selection, and/or adaption, it is possible to obtain higher ethanol producing strains from xylose (Figure 16). More recently, researchers at MIT have isolated mutants of *C. thermosaccharolyticum* which produce up to 3-4% (w/v) ethanol from xylose (Afeyan - personal communication).

A mixed culture consisting of two high-yielding ethanol tolerant strains was examined during fed batch fermentation of 80 g/ℓ of solka floc (439, 693). Initally *C. thermocellum* S-7 was added, followed by *C. thermosaccharolyticum* HG-4 24 hours later. From the 60 g/ℓ of substrate degraded, 25.3 g/ℓ of ethanol was produced (Figure 17). This amounts to 0.43 g ethanol/g solka floc consumed or ~85% of the theoretical yield based on the amount of substrate utilized. A fed batch mixed culture fermentation of corn stover (100 g/ℓ) resulted in only 37% of the substrate degraded and only 9.7 g/ℓ of ethanol (Figure 17). The increased acetate (possibly from enzymatic deacetylation of hemicellulose residues) reduced the overall ethanol yield to 0.26 g ethanol g substrate or ~52% of the theoretical yield based on the amount of corn stover consumed. The reduced yields were probably due to the potential toxicity of lignaceous components which are not present in solka floc (693).

Saddler and Chan (695) from Forintek, Canada, also examined *C. thermocellum* with both *C. thermosaccharolyticum* and *C. thermohydrosulfuricum* in co-culture fermentations on pretreated aspen and wheat straw fractions. Unfortunately, their efforts were met with little success.

The General Electric Corporate Research and Development (GE/CRD) Process also employed the mixed thermophilic culture of *C. thermocellum* and *C. thermosaccharolyticum* (518, 713). Strains *C. thermocellum* Q and *C. thermosaccharolyticum* ZC were tested for their ability to ferment low pressure SO_2-steamed hardwood directly to ethanol. A strain development program resulted in *C. thermocellum* Q5 which was able to grow in media containing 5% (w/v) ethanol (518). However, efforts to improve *C. thermosaccharolyticum* were unsuccessful. In their process, ethanol was continuously withdrawn from the fermentation broth to a separate vacuum distillation chamber (modified vacuferm), where it was subsequently distilled (518). The cells were then recycled and the lignin dried and used as fuel for the pretreatment step. Although preliminary 1979 ethanol cost analyses were encouraging, pretreatment, optimization and improvements in ethanol yields have to be made, before larger scale evaluation is justified.

d. *Clostridium thermocellum* and *Thermoanaerobacter ethanolicus*

Wiegel and co-workers (170, 441, 450) at the University of Georgia, have examined *T. ethanolicus* JW200 and *C. thermocellum* JW20 in co-culture fermentations of various cellulosics. They reported a greater yield of ethanol from the co-culture fermentations of filter paper cellulose over that of *C. thermocellum* alone (0.83 versus 1.52 moles ethanol/mole metabolized glucose-equivalent cellulose) (170). It appears that these higher yields of ethanol result when the pH is maintained at 7.5 (473).

This mixed culture system could also ferment a wide variety of cellulose-containing materials (steam exploded wood, acid hydrolyzed hardwood, bermuda grass, corn stover, wheat straw); however the ethanol yields were low (450, 473). Table 28 illustrates that *C. thermocellum* JW20 alone ferments steam exploded wood poorly and is inhibited by xylose and cellobiose. In contrast, in co-culture with *T. ethanolicus* JW200, the mixed culture effectively converted the available carbohydrates to ethanol and acetate (450). Furthermore, the addition of xylose and cellobiose resulted in more ethanol. Although final ethanol concentrations were still very low (<0.5% w/v), it is probable that these yields could be substantially improved by using strains with improved ethanol tolerance. In particular, the use of mutant *T. ethanolicus* JW200 Fe(4) which can produce up to 3-4% (v/v) ethanol from starch and is protected by a patent (441, 467), should be examined on cellulosics in co-culture with ethanol tolerant isolates of *C. thermocellum* (eg., strain S-7) (439).

e. General Electric Company Processes

The General Electric Company, Schenectady, New York, has described three mixed culture fermentation processes for the production of ethanol from cellulose. The GE/CRD process employing *C. thermocellum* and *C. thermosaccharolyticum* has been discussed in the preceeding section. Another process involves the thermophilic cellulolytic anaerobic gram negative *Sporocytophaga sp.* and an ethanol producing thermophilic gram positive variant of *B. stearothermophilus* (526). Presently, all attempts to grow this *Sporocytophaga sp.* in pure culture have been unsuccessful; growth is always accompanied by the *Bacillus* (528). This mixed culture was isolated from two sources; a freshwater mangrove swamp in Yucatan, Mexico, and from a decaying elm stump in

Schenectady (528). The process [which has been patented (526)] has been investigated under partial vacuum, and results in the production of ethanol and acetic acid at a weight ratio of about 1:1 (739). However, this culture does not utilize the hemicellulose portion. In an effort to improve the process, one project at Cornell University involved selecting for ethanol tolerant thermophilic *Bacillus spp.* which had the ability to rapidly grow and ferment various substrates including xylose (739). Jewell *et al.* (740) have recently initiated a study to evaluate the feasibility of alcohol production from agricultural wastes using an attached microbial film expanded bed reactor containing *C. thermocellum* and *Sporocytophaga*.

A joint venture between the University of Pennsylvania and the General Electric Company, (the Penn/GE Process) has also received much attention (741-745). Wood chips from pulp waste are pretreated to yield cellulose. *Thermonospora* enzymes hydrolyze the cellulose which is simultaneously fermented to ethanol by *C. thermocellum*. Other researchers have also reported thermophilic SSF processes employing the same organisms (665, 746). β-Glucosidase added to the fermentor, prevents cellobiose buildup that would otherwise inhibit the hydrolysis. Ethanol was removed continuously by mild vacuum to yield ~25% ethanol in the condensate (744). This process also planned to incorporate a recycling of the hydrogen gas generated during the fermentation in order to increase ethanol yields and reduce acetate production (713, 745). In a second stage, the residual hemicelluloses were fermented to acetone butanol by *C. acetobutylicum*.

In order to eliminate the second stage and to get the hemicelluloses fermented, an alternative process has been examined using *Thermonospora* with

C. thermohydrosulfuricum (742, 743, 745), *C. thermosaccharolyticum* (743, 746) or *T. ethanolicus* (745). The use of the latter bacterium also eliminated the need for β-glucosidase. However, even with ethanol tolerant strains, low concentrations of ethanol were obtained since growth of *T. ethanolicus* was found to be limited by the rate of cellulose digestion. To overcome this problem and to take advantage of the fact that cellulase is adsorbed by the cellulosic materials, Pye (745) developed a novel design. A continuous countercurrent tower for high temperature saccharification of cellulose was followed by the fermentation of the sugar stream in a packed bed containing immobilized *T. ethanolicus*. This design should theoretically reduce the amount of expensive enzyme needed, however, more research is required. Unfortunately, cost estimates at the time showed that it was not feasible to embark on this project (745).

f. Other Thermophilic Co-Culture Fermentations

Saddler *et al.* (427, 428) examined the potential of a co-culture fermentation employing *C. thermocellum*, and *Z. mobilis* or *Z. anaerobia*. *C. thermocellum* was cultured for 3 days at 50°C on 2.5% (w/v) cellulose. After the fermentor cooled down to 37°C, *Z. mobilis* was added. Although, the co-culture produced 9 times more ethanol than with *C. thermocellum* alone, the ethanol yields and productivities were still very low (<0.3% w/v) (427). In order to increase the glucose availability, β-glucosidase was added to the fermentor (428). After the first 24 hours, concentrations of ethanol increased. However, after the first day there was no change, as a result of the loss of activity of β-glucosidase at this temperature. Although ethanol values were low, results obtained on pretreated wood fractions were almost as good as those obtained on solka floc (428). Viikari and co-workers (422) obtained a higher conversion of cellulose to ethanol by increasing the enzyme in an SSF process employing *T. reesei* and *Z. mobilis*.

Biotechnology companies may also see potential gains from fermentation ethanol. Kendal Pye, president of Biological Energy Corporation, hopes to improve profits by ~40% by employing thermophilic bacteria (747). Leslie Glick, president of Genex, stated at an Energy conference that "one of his company's objectives was to construct a thermophilic organism capable of producing over 200 g/ℓ/h of ethanol at temperatures ≥80°C" (748). The high temperature of the fermentation and the CO_2 evolved would increase the rate of ethanol evaporation. This should allow for the continuous removal of ethanol by distillation at atmospheric pressure. In theory, the thermophile would be immobilized in a reactor; the bottom of which would serve as a still for fermentation of substrate and the top as a standard fractional distillation column (748).

3. Simultaneous Saccharification Fermentation Processes (SSF)

Simultaneous saccharification fermentation (SSF) involves the enzymatic saccharification of cellulose and the simultaneous yeast/bacterial fermentation of sugars to ethanol in the same vessel. The main advantages of such a system are (580, 586, 749, 750): 1) elimination of the need for separate fermentors for saccharification and fermentation 2) acceleration of the rate of cellulose hydrolysis since catabolite repression is minimized 3) elimination of enzyme losses in the feed from saccharifier to fermentor 4) because of the limitation of the initial cellulose concentration, the ethanol concentration never reaches a level which is inhibitory to the yeast or cellulase 5) yeast growth easily maintains anaerobic conditions necessary for saccharification and this eliminates the necessity for strict aseptic conditions.

Requirements for a successful SSF process are (580, 587): 1) high ethanol productivity, 2) high substrate and ethanol tolerance, 3) tolerance of the particulate properties of cellulose, 4) resistance to the lytic effects of cellulase, and 5) minimal effects of yeast proteolytic enzymes on cellulase. Most importantly, it is necessary that the fermentation system and saccharification system be compatible with similar pH, temperature, and substrate concentration optima. One problem associated with the SSF process is the different temperature optima for saccharification and fermentation. Since the optimum saccharification temperature is ~45-50°C, the use of thermotolerant yeast strains or bacteria would be desirable in applying the coupled SSF process for cellulose conversion to ethanol.

A few laboratories have screened for thermotolerant yeasts (yeasts able to ferment well at ≥40°C) suitable for an SSF process employing the cellulase of *T. reesei*. Gulf's University of Arkansas Process, employed *C. utilis* at 42°C (632). Lastic and co-workers (587) screened ten yeast strains for ethanol production in an SSF process using cellobiose as the substrate. Of the ten strains, only *C. lusitanae* Y-5394 was able to ferment at temperatures >40°C, but only at cellobiose concentrations ≤10% (w/v) (Table 17). Blotkamp *et al.* (586) examined six strains of yeast for an SSF process. *S. cerevisiae*

4132 and *C. brassicae* IFO 1664 produced the highest ethanol concentrations. Ethanol concentrations produced by *S. cerevisiae* after 24 hours were 0.24% (w/v) and 0.06% (w/v), at 40°C, and 45°C, respectively. Under similar conditions ethanol concentrations of *C. brassicae* were 0.19% (w/v) and 0.18% (w/v) at 40°C and 45°C, respectively. McCracken and Gong (21) examined an SSF process at 30-50°C employing *Candida sp.* HT4. It was found that final ethanol concentrations increased significantly when the temperature was raised from 30 to 40°C. An increase in temperature to 45°C, was found to have little effect, while 50°C significantly slowed down the rate of fermentation.

Ghose *et al.* (585) examined an SSF process at 40°C employing the cellulase from *T. reesei*, the β-glucosidase from *Aspergillus wentii*, and the yeast *Candida acidothermophilum* ATCC 20381. From 10% pretreated rice straw, ~58% of the cellulose was metabolized in 14 hours. This resulted in an ethanol concentration and productivity of 2.3% (w/v) and 1.25 g/ℓ/h, respectively. When the SSF process was operated under vacuum recycling, the ethanol productivity increased to 1.8 g/ℓ/h. By far the best results were obtained when the process was operated under vacuum recycling with intermittent substrate feeding. Under these conditions ~98% of the cellulose was utilized and 4.5 g/ℓ/h of ethanol were produced.

Mansoor *et al.* (580) recently evaluated *K. fragilis* CSIR Y326 for an SSF process at 40°C. They concluded that their thermotolerant yeast had all the desirable properties necessary for a successful SSF process. Although *K. fragilis* fermented slowly at 50°C, it had an optimum fermentation temperature on 4% glucose of 40°C. After 9 hours, all the sugar was utilized. Cell viability and ethanol productivity were hardly affected by 6% (w/v) ethanol or 32% (w/v) glucose. Furthermore, it had a similar pH optimum to *T. reesei* (pH ~4.5) and it produced minimal amounts of proteolytic enzymes.

4. Xylan Utilization

In contrast to cellulose, the utilization of hemicellulose by thermophilic anaerobic bacteria has not been studied in detail (170, 503). The observation that *C. thermocellum* could produce xylose from solka floc indicated that the hemicellulose portion could be broken down (676). Garcia-Martinez *et al.* (516) observed that although xylan did not serve as a carbon source for growth, it could be hydrolyzed when cells were grown on cellobiose. The production of xylan-degrading enzymes by thermophilic fungi has also been reported (751, 752).

Studies have shown that acid hydrolysis can inhibit utilization of the crude xylan fraction. A proper preparation of hemicellulose fractions can be obtained economically in high yields by steam explosion processes (452). The BFA Hamburg-process (753) yields a relatively pure hemicellulose fraction from hardwoods ranging from single sugars to 40 sugar units. The ratio of xylose to xylo-oligomers and xylo-polymers depends upon the source, the temperature, and time of incubation (470). For example, higher temperatures and longer steaming times yield a better substrate for higher ethanol yields and productivities in *T. ethanolicus* (452).

Recent studies of Wiegel and co-workers (452, 470, 754, 755) showed that *T. ethanolicus*, *T. brockii*, *T. acetoethylicus*, *C. thermohydrolyticum*, *C. thermosaccharolyticum*, and *C. thermocellum* grew on xylan, and produced ethanol, lactate, acetate and CO_2/H_2 as by-products. The kinetic pattern of the breakdown and utilization of the various fractions of xylan differed significantly among various bacteria. *C. thermocellum* first hydrolyzed the high oligomeric xylan fractions (xylose, n $\geq$6). The low xylan fractions (n = 2-5) were only utilized after 240 hours, whereas the monomer xylose was not even utilized after 300 hours. On the other hand, *C. thermohydrosulfuricum* and *T. brockii* used the xylose first, then the short xylan oligomers (n = 2-5), followed lastly by the high oligomeric xylan fractions (755). *T. ethanolicus*, *T. acetoethylicus*, and *C. thermosaccharolyticum* utilized the lower xylose oligomers (n $\leq$ 3) at about the same rate as glucose and xylose. The maximum ethanol yield obtained on xylan (40.2 mM in 72 hours) was with *T. ethanolicus* JW200 Fe(4) (470). Although the ethanol yields and degradation rates were low, these findings were nevertheless encouraging. Other unidentified thermophilic bacteria have also been reported to ferment xylan (Section V, 4).

VIII. CONSEQUENCES OF THERMOPHILIC FERMENTATION

1. Microbial Features

a. Growth Rates

According to the Arrhenius law, an increase in temperature speeds up chemical and enzymatic reactions. Therefore, it has been assumed that thermophiles would exhibit higher catabolic activities at temperatures optimal for growth. This should result in faster growth rates, shorter fermentation times, and higher productivities (11). However, studies relating to the Arrhenius equation have usually been performed on single species at temperatures below the optimum for growth. From the limited data available, it can be seen that thermophiles do not grow as rapidly as one would predict from the Arrhenius equation (29). This suggests that even though thermophiles may be optimally adapted to their environment, they are not able to make full use of their thermal environment. Ljungdahl and Sherod (185) concluded from some of their early work, that enzymes from thermophilic microorganisms have sacrificed part of their catalytic activity, in order to function at higher temperatures. Mohr and Krawiec (756) recently studied temperature characteristics and Arrhenius plots for psychrophiles, mesophiles, and thermophiles. Their observations indicated that the temperature-dependent specific growth rates of selected bacteria occurred in two patterns. For each pattern, the temperature characteristics varied in a regular but non-linear manner relative to optimum temperature.

Sonnleitner and Fiechter (13, 82) recently showed that a process can be accelerated more effectively by increasing the culture temperature to the optimum of a given bacterium, rather than by using a comparable microorganism from a higher thermotolerance level at a sub-optimal temperature. Although most thermophiles are generally fast growing bacteria, there are exceptions to the rule (82). This is because bacterial growth rates are dependent on specific kinetic and molecular properties of thermophilic enzymes (46). If metabolic rates were as rapid as might be predicted based on the temperature differential, it is probable that the available nutrients in the environment would not suffice to sustain growth (176).

b. Cell Yields

The general higher metabolic rates of thermophilic bacteria and thermotolerant yeasts at optimal temperatures are usually accompanied by lower cell yields (13, 44, 239, 757); however there are exceptions to this (758-760). Factors permitting yeasts to retain the same growth yield during cultivation at alternate temperatures are reviewed by Pozmogova and co-workers (758). Studies of *Candida spp.* in the chemostat indicate that cellular yields are less influenced by temperature than by changes in growth rates caused by changes in temperature in batch culture (761, 762). It seems that the high metabolic activity at high temperatures is responsible for the low cell yield. It is interesting to note that when *Thermophilus aquaticus* utilizes certain substrates completely and grows slowly, it yields final biomass levels similar to mesophiles (13). However, in continuous cultures where it metabolizes rapidly, it produces extremely low yields.

The lower growth efficiencies of thermophiles (expressed as biomass produced per mole of substrate utilized) are not due to a less efficient catabolism or to a lower production of ATP. At the higher temperature more energy is required for cell maintenance, (maintenance of intracellular pH, membrane stabilization, ionic strength, higher turnover of cell constituents, etc.) (11, 46, 763). It appears that at higher temperatures, the maintenance requirements may increase at a greater rate than that of the overall catalytic efficiency (46). It was also reported that lower cell yields were also due to energy dissipation by the nonviable fraction of cells (764).

Final cellular yields are not only a function of maintenance energy, but also depend on the energy available from substrates and how well the microorganism conserves this energy. Thus, low cell yields might be compensated, if a thermophile's metabolism was thermodynamically more favorable at the higher temperature, or if it had evolved a more efficient mechanism of energy conservation (760). Brannan and Caldwell (760) found that despite higher maintenance requirements of *Thermothrix thiopara*, its growth yield exceeded those of mesophilic sulfur oxidizers.

c. Ethanol Yields and Production Rates

In the case of ethanol production, it might be argued which parameter is more important; ethanol yield or production rate. Although this will depend on the specific process, it can be assumed that both are equally important. For a process to be economically viable, one needs to have a reasonable yield of ethanol produced in a reasonable time period. Some researchers report ethanol yield, others production rates, and most often other factors such as cell density, reactor volume, culture conditions, etc. are not standardized between laboratories. This makes it very difficult to make direct comparisons of fermentation potential between microorganisms. High ethanol concentrations ($\geq$6% w/v) substantially reduce distillation costs while high productivities permit the use of smaller fermentors resulting in lower capital costs.

Sonnleitner and Fiechter (13) recently concluded (after extrapolating some data from various papers) that contrary to expectations, thermophilic bacteria in general do not have significantly higher product formation rates than mesophiles. Although their results (Table 29) back up this statement, it can be argued that only very recently have the potential thermophilic ethanologens been discovered. Since essentially little research has been conducted with them, conditions for their optimization are still not known. A major factor responsible for the low productivity rates is the inhibitory effect of ethanol on the membranes and enzymes (Section IX, 2a). It is the author's belief that productivity rates and ethanol yields will be significantly improved when more alcohol tolerant strains and/or alcohol derepressed ADH strains are selected for.

2. Process Features

High temperatures affect many design parameters including the choice of microorganisms, substrates, products, and fermentor controls. An increase in temperature changes many physical and chemical properties of water (Table 30). These changes are generally reflected as advantages, although some disadvantages also exist in large scale production of fermentation ethanol.

a. Substrate Solubility

As a result of higher temperatures decreasing the viscosity, surface tension, and density, substrates become more soluble in aqueous phases (13). This would allow one to use higher substrate concentrations and as a result upgrade productivity, provided the osmotic pressure was appropriate for the organism. Osmotic pressure control has been used to improve growth of thermophilic microorganisms (765). In addition, the elevated temperature could allow the use of substrates which have a low solubility at ambient temperatures because substrate solubility is often a rate limiting step in fermentation (11, 46). This also leads to a decrease in the required energy needed to maintain homogenous conditions in the fermentation broth (i.e., decreases mixing costs) (11).

Ziffer and Iosif (766) recently showed that the dissipation of heat at high temperatures was a problem in very high concentration corn fermentations. High ethanol yields were obtained in both low (~17% w/v) and high (~37% w/v) saccharified mashes at 30°C. At 34.5°C, similar yields of ethanol were obtained with the low corn mashes, however, at this temperature decreased ethanol yields were obtained with the high concentration corn mashes. They concluded that the decreased ethanol yields were related to mass and/or heat transfer problems rather than primary toxicity. The lower alcohol yield could be a result of a decrease in cell viability accelerated by the increased intracellular ethanol and aggravated by the poor diffusion of intracellular ethanol away from the cell in viscous high corn media (766, 767). An additional contributing factor could also be the problem of poor heat transfer in the high corn medium, with possible highly localized maxima (766).

b. Stress on Hardware

Most studies with thermophiles have been conducted using only test tubes, Hungate-tubes, or shake flasks. Experiences with controlled laboratory scale or even large-scale bioreactors are rare. It was recently pointed out that the stress of the high temperature on biotechnology hardware should not be underestimated (13, 82). Leaching of trace elements or plasticizers from steel or peripheral equipment (eg., tubing) has been reported to cause serious inhibition of growth of thermophiles (13, 82, 768, 769). In addition, the performance of biosensors such as D.O. probes and temperature sensors, deteriorates more rapidly at high temperatures, producing drift or noise in the signals (13, 770). These problems require extra maintenance of the equipment for proper functioning (82). Perhaps, these queries will motivate researchers to improve existing equipment to better control biotechnological processes.

c. Removal of Volatiles

The increase in vapor pressure at high temperature results in evaporation of volatile products. Therefore, less energy is required for distillation of ethanol. In addition, ethanol can be continuously removed from a fermentation broth by various novel processes which offer potential savings (Section IX, 4a). In fact, unless special precautions are taken, the high vapor pressures at high temperatures (i.e., >60°C) can lead to the evaporation of ethanol from the fermentation broth (82). Therefore, the ethanol produced during thermophilic fermentations may actually be higher than the reported values.

The removal of ethanol may also lessen the inhibition of the fermentation by its accumulation. It is also a means of removing potentially inhibitory products from a culture, such as acetic acid (13). The higher vapor pressure can also lead to extensive evaporation of the liquid media, causing the concentration of undesirable reactants or products (82, 771). Although it is not a serious problem, it should not be ignored. Evaporation can be reduced by sparging with humidifed gas, adding sterile water, or by using efficient reflux coolers but these can be additional complex and costly procedures (13, 82, 771).

d. Contamination

A general belief is that thermophilic biotechnological processes would rarely become contaminated. However, the experiences of scientists working with thermophilic bacteria show that their cultures often become contaminated (13, 46, 82, 441). Highly resistant spores from organisms like *B. stearothermophilus* or *C. thermohydrosulfuricum* are often found in biomass, and as contaminants. This may reflect Brock's (60) statement that "the quality of aseptic techniques of people working with thermophiles deteriorates with time, sometimes with disappointing results". However, Hyun and co-workers (497) reported that cultures routinely became contaminated despite cautious use of vigorous aseptic culture techniques. They suggested a minimum autoclaving time of 30 minutes for preparation of materials to grow thermophilic bacteria. In addition, *C. thermosaccharolyticum*, *C. thermhydrosulfuricum* and *B. stearothermophilus* were recently shown to be inhibited by antiseptic substances (772).

In comparison to cultivation of mesophilic bacteria, however, the requirements of sterility are less stringent for the growth of thermophilic bacteria, especially faster growing species. Operation of fermentors without sterilization for slower growing microorganisms, may require an additional step (eg., manipulation of culture parameters). Furthermore, fermentor operation at high temperature prevents accumulation of known bacterial and viral pathogens (11, 46, 773); an advantage especially in the bioconversion of municipal wastes. Among the thermophilic and thermotolerant fungi, animal pathogens may exist (774) and thermophilic *Actinomycetes* may be a causative agent for hypersensitivity pneumonitis in industrial workers in cellulose conversions (775).

e. Gas Solubility

Higher temperature causes a decrease in the solubility of gases like oxygen. For example, at optimal temperatures of *T. ethanolicus* (~67°C), the solubility of oxygen in the medium is less than 20% of that at 30°C (11). This decrease in oxygen solubility favours anaerobic fermentation and is also responsible for the lower cellular yields seen at high temperature (776). Until recently, it was unknown whether or not the D.O. in a culture might become limiting under particular conditions of aeration and agitation for a thermophilic microorganism (83, 770). It has been shown that the overall oxygen transfer coefficient K_L, increases with temperature, i.e., less oxygen is transferred from the bubble to the liquid phase resulting in lower oxygen solubility (82, 770). However, because of the broth's lower viscosity (C_s) at high temperatures, the volumetric mass oxygen transfer coefficient

(K_La) also increases i.e., oxygen is taken up faster by the cells. Owing to opposite dependencies between K_La and C_s, the maximum rate of oxygen supply remained fairly constant (770). It should be noted that this relationship is not linear in very viscous solutions (i.e., >50 cpoises) (771). Aiba *et al.* (770) concluded that the potential capacity to supply oxygen under given conditions of aeration and agitation seemed nearly independent of temperature. However, Aiba's conclusions were made studying only the facultative anaerobe, *B. stearothermophilus*. It still remains to be seen whether high performance bioreactors will be needed for cultivating thermophilic aerobes. With this in mind, the specific oxygen uptake rate of thermophilic aerobes tends to be higher than those of comparable mesophiles (13). In addition, the critical oxygen partial pressure may also be higher for thermophilic bacteria over mesophilic bacteria (13). Since oxygen supply to organisms can be increased with pressure, Romanelli (778) examined the effects of pressure on the fermentation of cellulose by thermophilic fungi.

It should also be noted that the solubility of other gases such as H_2 and CO_2 will also decrease. This may or may not be an advantage depending on the process and the organisms. A high concentration of hydrogen gas inhibits the growth of *T. ethanolicus* (59), while it favours ethanol formation in *C. thermocellum* (685).

f. Heating and Cooling

During fermentation, the metabolic activity of yeasts result in the exothermic production of heat, eg., 140.2 cal/g glucose for yeast fermentation (560).

Without temperature control, this rise in temperature continues until the temperature of the medium becomes intolerable for growth and activity. This often results in incomplete fermentation and thus low fermentation efficiency (592). Eventually, the culture enters a stationary phase, the fermentation slows down, stops, and the cells die. Usually this occurs at temperatures ~35°C, however it varies depending on the strain. In the absence of cooling, heat can be removed from a fermentor in two ways; through the walls of the vessel by conduction and radiation, and by evaporation caused by the evolution of CO_2 gas saturated with water (363).

In many fermentations, this rise in temperature is controlled by the use of energy intensive heat exchangers, or by circulating cooling water through coils suspended in fermentation vessels (363). For cooling water to be effective, the water temperature must be substantially colder than the operating temperature of the fermentor. With most fermentations operated at approximately 30°C (usually up to 37°C max.) and an average cooling water temperature of 20°C, there exists only a small driving force for heat removal (572). This problem is further magnified with large scale operations. As the size of the fermentor is increased, the amount of surface area available for heat transfer per unit volume decreases (572, 779). This decrease in heat loss necessitates more substantial investments for cooling (91).

In warm and humid tropical climates, the average cooling water temperature is so high that it often becomes imperative to operate plants at reduced capacity or shut them down completely during summer months (592). One alternative is to provide costly refrigeration to lower the temperature of the cooling water. Another way of minimizing this problem, is to operate the fermentor at higher temperatures and thus provide a greater driving force for heat removal and thus minimize cooling costs. In this regard, thermotolerant yeasts would be advantageous in many warmer regions of the world. This is exemplified by the fact that many of the selections for thermotolerant yeasts have been conducted in countries like India, South Africa, Cuba, Phillipines, etc. (Section VI).

It is thought that higher metabolic rates of thermophilic organisms might result in the production of enough heat to sustain the high temperatures needed for their fermentation (46). In fact, this might even necessitate some cooling. On the other hand, due to the higher heat dissipation in the fermentor at elevated temperatures, extra heat or thermal insulation might have to be added to the system to maintain the desired temperature optimum. In either case, a lot less energy will probably be required to keep the vessels at the desired elevated temperatures.

Until recently, simple and reliable methods were unavailable for predicting heat balances; the net amount of heat produced in a fermentor as a consequence of metabolism. Nevertheless, as early as 1914 Brown (555) predicted that as the initial temperature was increased from 5 to 40°C, there was a greater potential rise of heat in the fermentor. Cooney *et al.* (780) in 1968 devised the first simple reliable procedure for measuring the rate of heat production from a fermentation. They measured the rate of temperature rise in the fermentation broth when the temperature controller was turned off. They found that the rate of heat production during metabolism was directly proportional to the rate of oxygen consumption. The proportionality constant was independent of the growth rate and cell density, and only slightly dependent on the substrate and the type of organism (bacteria, yeast, or mold) investigated. The heat load (calculated by multiplying the oxygen demand by 0.12 k cal/m mole oxygen), was useful for predicting the amount of heat generated from the fermentation (780). Microcalorimetry was also a suitable method for measuring the heat produced in microbial processes (781).

Cooney and co-workers (780) finding on the direct heat oxygen correlation were also confirmed by many other researchers who examined the heat produced during fermentation under slightly different conditions i.e., oxygen limited, stirring, addition of gases, computerized program, etc. (781-786). However, this direct correlation was no longer valid when yeasts formed products such as ethanol (784, 787). This is probably because more heat was produced during aerobic respiration than during fermentation, since the former process makes more energy available. Due to limited capabilities to utilize this energy for growth and maintenance, a significant amount is released in the form of heat (788). This can also partially explain why less heat is generated with high cellular yields.

Various factors will affect the amount of heat released by the cell. Heat is produced as a result of cells both synthesizing ATP and breaking it down (789). The amount of heat released also depends on the efficiency with which the cells can utilize the substrate; this is reflected in the cellular yield coefficient (572). The higher the cellular yield, the lower the amount of heat generated (572, 790). The quantity of heat released per gram of microorganism will thus vary with different substrates. The fermentation of pentoses results in the release of larger quantities of heat than hexoses

(791). In addition, much more heat is produced during growth on fully reduced compounds such as hydrocarbons than on more conventional substrates such as glucose (572). Furthermore, as one might expect, the higher the substrate concentration, the more heat produced (363).

A considerable amount of heat is also produced by endogenous metabolism, i.e., in the absence of a carbon or energy source (792). This is presumably due to polyphosphate de-phosphorylation (789). Other factors such as age of the cells and cell numbers will also affect the amount of heat produced (792). For example, resting cells produce less heat. Baker's yeast stored for 2 weeks at 4°C looses the capacity for endogenous heat development (789). In a similar manner, traces of azide or dinitrophenyl, can also eliminate endogenous heat (789).

For strictly reducing cooling costs, an alternative strategy to selecting for thermotolerant microorganisms would be to select for organisms which produce less heat. It can be seen from the above that this can be obtained to some degree by manipulating culture conditions, age of cells, substrate levels, etc. Another means of achieving this might be by selecting for organisms which produce less ATP or break it down more slowly (eg., less ATPase activity). Such mutants and optimal conditions should be more easily obtained with the advent of the simple means of measuring heat production discussed earlier.

In view of the potential industrial uses of thermophilic bacteria, it is imperative to learn more about heat production of thermophiles during fermentation (793). Rothbaum (794) examined the heat output of thermophiles occurring on wool by crude techniques. Aiba and co-workers (779) recently examined the significance of temperature control of a mesophilic (*E. coli* - T_{opt} ~41°C), thermophilic (*B. caldotenax* - T_{opt} ~64°C), and extremely thermophilic bacterium (*T. thermophilus* - T_{opt} ~72°C). With the thermophilic organism, they found that some heat was lost during the early period after the start of the fermentation. In order to keep the broth temperature at the optimal level, minimal energy was initially required for heating (~4% of the energy required for cooling). After this, the thermophilic bacterium produced so much heat, that cooling was required to keep the temperatures optimal for fermentation. However, the amount of cooling required for the thermophile was only 30% of that for the mesophile. The extreme thermophile required ~15% more cooling water than the thermophile. Aiba and co-workers (779) attributed this to the extreme thermophile's faster growth rate at the optimum temperature causing a greater rate of heat evolution. Although Cooney *et al*. (780) (as discussed earlier), reported that the rate of heat production was independent of growth rate, they never examined fermentation by a thermophilic organism.

Without temperature control of the culture, the fermentation temperature was unbalanced i.e., no balance between heat evolution and heat loss (779). Although the results were strictly based on calculations and assumptions, they show that indeed some heating and cooling may be necessary for thermophilic fermentations. Nevertheless, especially after sterilization of the medium, where heat loss is minimized (11, 82), thermophilic fermentations can keep expenditures for cooling or heating to a minimum.

IX. APPROACHES TO ENHANCE THERMOPHILIC ETHANOL FERMENTATIONS

1. Physiological Aspects

a. Thermoadaptation and Nutritional Requirements of Thermophilic Bacteria

Thermoadaptation, another means to increase the growth or fermentation temperature of a bacterium, can be accomplished by continuous transfer to progressively higher cultivation temperatures. This approach has been successful in raising culture temperatures of various microorganisms, but this is usually only temporary and to a limited extent. Similarly, supplementation of nutrients to various media sometimes permits growth at higher temperatures, but this too is only to a certain limit. Recent reviews have been published examining strategies of microbial adaptation to extreme environments (45, 195, 206).

The development of thermophilic species after long periods of growth at elevated temperatures may represent the gradual accumulation of mutations which enhance survival and growth under these harsh conditions (49). However, it is quite possible that in some thermoadaptation programs, thermotolerant contaminants are acquired up in the process (4). Therefore, it would seem advisable to conduct studies with genetically marked strains.

The most remarkable report of thermoadaptation in the literature is Dallinger's detailed account in 1887 (795). During approximately a seven year time period, the growth temperature of 3 species of flagellates was significantly increased, by slowly increasing their incubation temperature from 15 to 70°C. The experiment ended when the flasks tipped over! Although these results have been questioned, no attempt has been made to repeat these experiments for obvious reasons (34)!

Campbell (796) demonstrated that an obligate thermophilic bacterium could be facultatively grown at 36°C, when certain nutrients were added exogenously. This observation was confirmed shortly thereafter by others (797). Bausum and Matney (2) reported that some facultative thermophilic *Bacillus spp.* were rapidly killed when the growth temperature was suddenly raised from 37 to 55°C. However, cell death was avoided if the bacteria were previously allowed to adapt at an intermediate temperature for a limited period of time before the shift to high temperature. In this way, Jung *et al.* (531) were able to convert obligate thermophilic strains of *B. stearothermophilus* into mesophilic variants and back again into their original thermophilic forms. In a similar manner, it was demonstrated that the heat resistance of bacteria depended on the temperature of incubation prior to heat treatment (798, 799).

Dowben and Weidenmuller (49) adapted *Bacillus subtilis* (T_{max} ~55°C) to grow at elevated temperatures as high as 72°C; however, when the adapted bacteria were subsequently grown at 37°C, the ability to grow at high temperatures was lost and the bacteria had to be re-acclimatized. These investigators noted that adaptation was accompanied by an increase in cell volume and protein content, while the nucleic acid content hardly changed. It should be noted that not all bacteria can be easily adapted to growth at high temperatures. For example, Casman and Rettger (800) were unsuccessful in their attempts (over the span of a year) at adapting ten spore-forming mesophilic bacteria to elevated temperatures.

It was demonstrated that the thermostability of various enzymes from *B. stearothermophilus* (eg., α-amylase, pyrophosphatase, glucokinase, etc.) varied with the cultivation temperature (173, 234, 260, 801-804), i.e., these enzymes were thermostable when the *Bacillus* was grown at high temperatures (~55°C) and thermolabile when the *Bacillus* was grown at mesophilic temperatures (37°C). Haberstich and Zuber (490) reported a mixture of thermostable and thermolabile enzymes at intermediate temperatures. In their study, differences were found between *B. stearothermophilus* and *B. caldotenax* in the adaptation process. The adaptation process seemed to correspond to the physiological and environmental requirements of these two *Bacilli*. *B. stearothermophilus* which lives in an environment with slow temperature change, adapted only when an intermediate temperature of 46°C was used, while with *B. caldotenax*, direct transfers and conversions could be made. With this latter bacterium, adaptation occurred during the lag period whose length depended on the difference in the preculture and the culture temperature (490). Their experiments also showed involvement of protein synthesis in the adaptation process.

In some of the studies discussed above (490, 531), thermoadaptation and/or conversion was dependent upon inclusion of brain heart infusion (BHI) in the medium. Similarly, it was shown that the thermostability of LDH from *B. caldolyticus* was increased in the presence of BHI added to the growth medium (280). The component(s) in BHI that may facilitate thermoadaptation are not yet known, and hence, should be defined. However, the requirement of BHI for *B. caldotenax*, has been shown to be a result of an auxotrophic requirement for methionine and biotin (16).

As previously mentioned (Section V), most thermophilic ethanologens (eg., *T. ethanolicus* and *T. brockii*), have been reported to require growth factors that are usually supplied in the form of yeast extract. However, tryptone, peptone, and other frequently used complex substrates have also sometimes been found to suffice. Few attempts have been made to substitute yeast extract with cheaper nitrogen sources (eg., corn steep liquor, stillage, etc.) (466).

The nutritional requirements of thermophilic bacteria have been examined by a number of investigators (524, 805-809). Most studies have been conducted using *B. stearothermophilus*. In general, thermophilic bacteria tend to have complex nutritional requirements (eg., essential amino acids and/or vitamins) with increasing temperature. However, some strains of *B. stearothermophilus* also require additional cofactors at the lower temperatures (237).

Although there is little evidence that thermophiles synthesize unique stabilizing factors, studies indicate that a variety of interactions with cellular components (substrates, cofactors, metal ions, etc.) contribute to thermal stability. The role of cations and other extrinsic factors in enhancing thermostability has been previously discussed (Section III, 2). The composition of lipids in contrast to proteins is changed in almost all microorganisms. The means by which microorganisms alter the fluidity of their membranes by changing their fatty acid contents in response to higher temperatures has also been discussed in an earlier section (Section III, 3).

b. Metabolic Control of Thermophilic Bacteria

A fundamental understanding of metabolic regulation in thermophilic ethanologens is essential in order to increase their ethanol productivity, rates, and yields. Regulation of end product formation in mono-cultures (intraspecies control) and co-cultures (interspecies control) by thermophilic anaerobic bacteria is complex and still poorly understood. However, factors that control the flow of carbon and electrons as well as tolerance to specific products are becoming more evident in certain ethanologens.

All thermophilic ethanologenic bacteria described to date metabolize hexoses and pentoses to pyruvate via the Embden-Meyerhof-Parnas Pathway (27). Figure 18 illustrates the hetero-ethanol fermentation which results in formation of multiple products (ethanol, acetate, lactate, and CO_2/H_2) from pyruvate for a typical anaerobic thermophilic ethanologen (27, 48, 503). Pyruvate can be metabolized in 2 ways: reduction to lactic acid via a FDP-activated NAD-dependent LDH, or cleavage to acetyl CoA and CO_2 by a phosphoroclastic reaction. Acetyl CoA so-formed, can be metabolized in 2 ways: reduction to ethanol via a NADH-dependent acetaldehyde dehydrogenase and ADH, or hydrolysis to acetic acid via phosphotransacetylase and acetate kinase. Although acetate production provides extra ATP, it does not reoxidize the reduced NADH or ferredoxins produced during glycolysis. This regeneration is channeled through the oxidoreductase and hydrogenase systems resulting in H_2 production. Ethanol and lactate production, on the other hand, allows the reoxidation of the reduced pyridine nucleotides.

Thermophilic ethanologens that employ the same glycolytic pathway, produce the same end products but in different ratios. For example, for *C. thermocellum*, *C. thermohydrosulfuricum* and *T. brockii*, grown on similar cellobiose media, the ethanol/acetate ratios were 1.2, 17.7, and 4.7 respectively (139). These differences have been shown to be related to the specific activities and regulatory properties of the enzymes controlling electron flow.

The general factors influencing metabolic regulation in thermophilic ethanologens are summarized in Table 31. Ethanol yield appears to be mostly related to the activity and regulatory properties of: ADH, especially enzyme tolerance to ethanol and NAD; ferredoxin-NAD(P) oxidoreductases; and FDP-activated LDH. It would be expected that specific changes in culture conditions influence the activity and regulatory properties of these enzymes, and thereby influence the ratio of ethanol, lactate, acetate, and CO_2/H_2.

Metabolic control of end product formation in the thermophilic ethanologens has been best studied in *T. brockii* and *C. thermocellum* and is reviewed extensively by Zeikus *et al.* (27, 48, 139). In addition, Ljungdahl and Wiegel's group in the University of Georgia have recently published interesting results on metabolic regulation of *T. ethanolicus*. The metabolic control of these three bacteria will be discussed in the following section.

i. *Thermoanaerobium brockii*

T. brockii was considered to be unsuitable for ethanol production mostly due to low ethanol and high lactate yields (449, 460). Research on metabolic control in *T. brockii*, may subsequently make this thermophile exploitable for industrial alcohol production. In *T. brockii* fermentations, the flow of electrons from energy source to end products is interconnected by key reversible oxidoreductases and the common electron carriers, ferredoxin, NAD, and NADP (see Figure 19) (461, 462). Zeikus' group showed that this flow could be regulated and reversed by the addition of exogenous electron donors and/or acceptors (139, 462). *T. brockii* has both ferredoxin-NAD and NADP-oxidoreductase activity. It is thought that the former activity functions in a catabolic role by transfer of electrons derived from pyruvate to reduce NAD (27). It has been suggested that NADP-oxidoreductase has an anabolic role by supplying NADPH for biosynthetic purposes (48).

It was mentioned previously that growth of *T. brockii* was totally inhibited by 1 atm of H_2 (48). This was explained by the reversal of electron flow from H_2 (the electron donor) to reduction of NAD which was needed to perform the key oxidation of glycolysis (i.e., G3P dehydrogenase) (48, 139) (Figure 19). The growth inhibition by H_2 can be reversed by the addition of a variety of electron acceptors such as acetone, thiosulfate, and sulfite (48); H_2 is no longer a metabolic inhibitor because the reduction of acetone to isopropanol via acetaldehyde NADP reductase allows for the regeneration of oxidized pyridine nucleotides (462, 463). The addition of exogenous acetone to *T. brockii* batch fermentations results in an increase in growth rate and acetate, and a decrease in lactate, ethanol, H_2, and intracellular FDP (Tables 32, 33) (27, 48, 462). Furthermore, as a consequence of the increased oxidation rate of reduced cellular electron carriers, H_2 and acetone are almost stoichiometrically converted to isopropanol (27, 139). The addition of acetone to a chemostat culture of *T. brockii* however did not further speed up growth and it reduced product yields (460).

The methanogen *Methanobacterium thermoautotrophicum*, can act as a biological electron acceptor, as a direct consequence of hydrogen consumption (139, 810-812). Although monocultures of *T. brockii* or *M. thermoautotrophicum* alone cannot grow on ethanol, the mixed culture can, and acetate and methane form. This is because of the reversible ADH of *T. brockii* (463), and the ability of the methanogen to maintain low H_2 pressure (27, 48). Anaerobic growth on ethanol is thermodynamically feasible only when the H_2 pressure remains very low (813, 814).

Higher lactate yields in this ethanologen were in part related to the flow of electrons from pyruvate to lactate, but in addition, to the regulatory properties of the FDP-activated NAD-dependent LDH (461). Therefore, factors that lower the intracellular level or influence the activity of FDP should result in more ethanol formation. Table 33 illustrates that the amount of ethanol produced by *T. brockii* fermentations is indirectly correlated with the intracellular FDP content. The ethanol/lactate ratios on glucose and starch, were 0.7 and 2.65 respectively. This correlated well to the intracellular FDP concentration which was ~26 times higher during growth on glucose as compared to starch (462). Perhaps growth on the more readily metabolized energy source (glucose in lieu of starch), or on high concentrations of sugars, results in higher intracellular FDP levels, and hence more lactate and less ethanol (139, 459). It is thought that use of slow substrate feed rates and maintenance of neutral to alkaline pH should enhance ethanol yields (459). Other culture manipulations (temperature and age of cells), have resulted in no significant change in end product ratios (459).

ii. *Clostridium thermocellum*

C. thermocellum produces less ethanol and lactate, but more H_2 when cultivated under similar cultural conditions to *T. brockii*. This is related to the high hydrogenase activity and the absence of electron flow from reduced ferredoxin or from NADPH to lactate or ethanol. The latter is due to the apparent absence of ferredoxin NAD-reductase and NADP-linked acetaldehyde reductase activities (27).

It was previously discussed (Section VII, 1d, iii) that increased H_2 in the broth inhibits acetic acid formation of *C. thermocellum* YS, and removal of hydrogen by stirring favours acetate formation (685). These results are due to the regulatory effects of H_2 on the metabolic pathway (503, 685). An increase in H_2 concentration lowers the H^+/H_2 redox potential which in turn inhibits H_2 formation via the reversible hydrogenase reaction (685). As a result less acetate is produced (which is dependent on H_2 formation) and more electrons are channeled through the ferredoxin-NAD reductase system to reduce more acetyl CoA to ethanol. Similarly, when the medium is stirred, the trapped H_2 escapes, and the more energy efficient pathway leading to acetate formation is favoured. Lamed and Zeikus (461) reported that the addition of H_2 had no effect on *C. thermocellum* strain LQR1. This may be due to the absence of detectable ferredoxin-NAD(H)-reductase activity (503). However, Su *et al.* (685) observed that vigorous stirring of *C. thermocellum*

LQR1 still resulted in a 3-fold decrease in the ethanol/acetate ratio.

C. thermocellum, also has a FDP-activated NAD-dependent LDH (461). However, compared to *T. brockii,* lactate is only a very minor product. This is in large part due to the lower intracellular levels of FDP produced by *C. thermocellum*.

Differences in regulatory features of ADH activity between these two *Clostridia* are also responsible for the different product ratios (48). Unlike *T. brockii*, the ADH of *C. thermocellum* is only unidirectional and only catalyzes the reduction of acetaldehyde to ethanol. Enzyme activity has been shown to be NAD-linked and it is significantly inhibited by very low concentrations of NAD and ethanol.

iii. *Thermoanaerobacter ethanolicus*

Although this bacterium ferments low sugar concentrations ($\leq$1% w/v) nearly quantitatively to ethanol, wild type strains are inhibited by ~.6% (w/v) ethanol (450). Above ~1% substrate concentrations, a shift in the fermentation occurs with the production of more acetate and lactate at the expense of ethanol. The inhibition of the fermentation appears to be related to metabolic controls at the enzyme levels, notably, ADH, and LDH.

The FDP-activated pH-dependent LDH is NAD-dependent, but a slow rate of pyruvate reduction is also seen with NADPH (815). At pH $\leq$9.5 it calatyzes essentially an irreversible reduction of pyruvate to lactate (815). At pH 3.5

to 6.5, LDH is active without FDP. Above pH 6.5, the enzyme has high activity but only in the presence of FDP (441, 816). One can postulate that in media containing high substrate concentrations and/or at high pH, especially in the presence of readily metabolizable compounds, the amount of FDP inside the cell is increased (462). As a result this could lead to an activation of LDH and more lactic acid formation.

There appear to be 2 ADH's in *T. ethanolicus* (816, 817). One ADH (2°) has a molecular weight of 172,000 and consists of four subunits each of which contain 4 zinc atoms (816). Although ethanol is a substrate for this ADH, the rates of the reaction are faster with secondary alcohols. The enzyme is specific for NADP and the activity with NAD is less than 0.5% of that with NADP. Results indicate that both $NADP^+$ and ethanol may serve as effective substrate inhibitors in the reduction of acetaldehyde to ethanol (Figure 20). The activity of this enzyme is also increased with increasing pyruvate concentrations; pyruvate may bind to the ADH and stimulate its activity (441, 450). The kinetics of ADH with ethanol and acetaldehyde are sigmoidal suggesting that this enzyme may be subject to allosteric control with ethanol as the effector (59). Another ADH (1°) has recently been found (817). The subunits contain no metals, and the enzyme works better with 1° alcohols such as ethanol (817, Ljungdahl - personal communication). However, the regulatory relationship of the two enzymes is still unknown (817).

Figure 20 clearly illustrates that when the ethanol concentration is high, the ADH responsible for producing alcohol is turned off. This regulatory system in addition to the known effects of ethanol on the membranes may be responsible for low ethanol yields at high substrate concentrations. If so, it would seem more reasonable to isolate ADH deregulatory mutants rather than attempt to adapt these bacteria to higher ethanol concentrations (59).

In efforts to overcome these regulatory problems, Ljungdahl *et al.* (59, 441), have exposed the wild type *T. ethanolicus* JW200 to UV-light and to media deficient in iron, then they supplied excess $FeSO_4$. The former treatment resulted in *T. ethanolicus* JW200 L, while the latter, a series of mutants referred to as *T. ethanolicus* JW200 Fe(n). Both these treatments have successfully produced mutant strains which ferment starch to ethanol at high concentration and with less acetate production (59) (Figure 21). Although mutants of *T. ethanolicus* could tolerate up to 10% (v/v) ethanol without adaptation, these mutants could only produce up to 3% (w/v) ethanol (441). This again suggests that they may have a regulatory problem rather than an alcohol intolerance problem. The wild type on the other hand, could grow in media containing 10% (v/v) ethanol, however this was only after adaptation through stepwise exposure to higher ethanol concentrations. Table 34 illustrates the ethanol formation rates of resting cells of *T. ethanolicus* and its mutants.

Carreira *et al.* (815) found significant differences between the kinetic properties for the ADH's isolated from the wild type and mutants. The Km values indicated that NADP and ethanol were less effective as product inhibitors with the mutant enzymes than with the wild type ADH. Furthermore, the 1° ADH in the mutant Fe(4) appeared to have higher enzymatic activity (817). From studies with *C. thermohydrosulfuricum* strains, Zeikus *et al.*

(492) similarly concluded that ethanol intolerance in wild type strains is ascribed to the ethanol-dependent inhibition of ADH. It was observed that the ethanol tolerant mutant of *C. thermohydrosulfuricum* lacked the NAD-linked ADH found in the parent (492). More recently, Zeikus and co-workers isolated ethanol deregulatory mutants of *C. thermohydrosulfuricum* which were capable of producing up to 3-4% (w/v) ethanol from sugars (Zeikus - personal communication).

An enzyme exhibiting both inorganic pyrophophate (PPi) and ATP acetate kinase activity, has recently been purified from *T. ethanolicus* (818). Since *T. ethanolicus* has been found to utilize PPi as an energy source, it was suggested that this kinase may be involved, and possibly PPi could be used to modify the fermentation products. In this regard, the presence of PPi doubled the amount of ethanol produced from starch, in an unidentified thermophilic bacterium (isolate RB-3) (818).

c. Effect of High Temperature on Yeast Metabolism and Cellular Composition

The metabolic activities of yeasts are greatly affected by the temperature at which they grow. The influence of temperature on yeast physiology has been previously reviewed (819-821). Temperatures above the optimum lower the growth rate, oxygen solubility, and also change the cellular composition of yeasts. It is also known that under oxygen limited conditions, yeasts require nutritional supplements for growth, such as, unsaturated fatty acids (822). In batch culture studies (172), it cannot be established whether the changes in cellular composition at higher temperatures are a direct result, or an indirect consequence of a decrease in growth rate or oxygen availability. Assessment of the effect of high temperature on cells can best be carried out only in a chemostat which can supply oxygen at a constant rate. In fact, chemostat studies have shown that factors originally thought to be due to high temperature (eg., low cell yield), were really due to changes in specific growth rates and oxygen supply and/or nutrient requirements (761, 762, 776, 823).

An increase in temperature does not inhibit substrate uptake nor does it significantly alter enzyme levels (568, 824). Although energetically less efficient metabolic pathways replace energetically more efficient ones at high temperature (568), there is an increase in the content of intracellular ATP (825). This is true for both yeasts as well as bacteria (568). This is in part due to the inhibition of the energy intensive aerobic oxidative processes and to a decrease in ATPase activity (825). As a result of the shift in pathways, volatile acids, ethanol, and keto acids (eg., α-ketoglutarate, pyruvate) accumulate (824). The accumulation of the latter can be explained by inhibition of enzymes in the TCA cycle (i.e., α-ketoglutarate dehydrogenase and glutamate dehydrogenase), and by the decreased rate of assimilation of the keto acids (568, 824, 826). At higher temperatures, there is a marked decrease in the respiratory capacity of yeasts (826) as well as an enhanced rate of fermentation (827). However, it has been shown that high temperatures exceeding the optimal can increase the respiratory rate of microorganisms (828).

It has been documented that elevated temperatures induce respiratory-deficient yeasts or petite mutants (829-831). Induction of petites at high temperatures occurs more readily in early log-phase cultures (832). Other investigators also showed that ethanol induced petites (833, 834). These results suggest that mitochondria may not play an important role in imparting thermotolerance in yeast. However, mitchondria may play an important role in thermal death in the presence of ethanol (Section IX, 2c).

Parada and Acevedo (835) have recently examined the relationship of temperature and RNA content to the specific growth rate in *S. cerevisiae*. Various studies by researchers in the U.S.S.R. have examined the effect of high temperature on the cellular composition of yeasts in chemostats. Growth of thermotolerant yeasts at high permissive temperatures results in a change in their biomass composition (568). The yeasts decrease in volume partly as a result of a decrease in cellular protein, RNA, DNA, and amino acids (303, 576, 836-839). As a result of excretion by the yeasts there is an accumulation of a number of metabolites in the culture filtrate (840). Although the extent of free amino acids in the medium increases, this amount is negligible and does not significantly decrease intracellular pools (841). Cells grown at temperatures above the optimum also exhibit a number of changes in their ultrastructural organization. There is an attenuation of their membrane systems; nuclei are frequently lobed, and there is the destruction of the mitochondrial cristae (842). At elevated temperatures some researchers have shown that yeasts form long strands (563). This is possibly due in part to temperature inhibition of bud detachment (827, 838, 843).

d. Factors Affecting Yeast Growth and Fermentation at High Temperature

It was previously mentioned that various factors can affect yeast growth at elevated temperature (Table 1). Many of these have been discovered from studies on heat resistance of yeasts, most often in beer and wine (556, 844-849). As early as 1889, Kayser (850) conducted heat resistance studies on yeasts and reported that various factors (eg., age of cells) can influence the thermoresistance of yeasts.

Thermal-killing can vary with the stage of growth, the position in the cell cycle, or the composition of the media (851, 852). A number of investigators have reported that exponentially growing yeasts are more heat-sensitive than older stationary cells (845, 846, 853, 854). It also follows that yeasts will survive temperatures under more favourable conditions. Pozmogova *et al.* (855) showed that the detrimental effects of higher temperature were greater at less favorable pH. Similarly as might have been expected, higher initial cell numbers had greater protective effects at elevated temperature (845). Various studies have also shown that yeasts incubated at higher temperatures have more complex nutrient requirements (19, 562, 856-859). In a similar manner, the synergistic enhancing effect of various chemicals (eg., sorbic acid) on thermal injury has been studied (860, 861).

Rudenok and Konev (862) claimed that thermotolerance increased with cell number due to the release of protective substances from the cells. They found that supernatants obtained from heat-treated, concentrated cultures released non-polar or uncharged hydrophobic amino acids. The hypothesis was that after heating, there were functional and structural modifications of the membrane which resulted in the release of these "thermoprotectors" from the cells. It was thought that these stabilized the membrane and enhanced the thermotolerance of the yeasts (862). A greater protective effect of higher cell densities may simply be due to an increase in the number of viable cells left in the population after killing. The amino acids released by the cell may quite possibly serve no protective role, but rather, be just a consequence of growth at high temperatures (Section IX, 1c). However, it has been shown that non-polar amino acids have a protective effect on yeasts against heat (846, 863).

It is possible that specific unknown "thermoprotectors" in yeast cells could stabilize a variety of macromolecules by altering intermolecular bonding. Evidence already suggests that yeast glycosylated proteins are more thermostable than non-glycosylated ones (864). A mechanism of thermotolerance for a general thermoprotector would require that the thermoprotectors accumulate at key sites within the cell as a result of biosynthesis, a change in compartmentalization, or uptake from the medium (865). Although research in this area is lacking, it is possible that specific proteins, carbohydrates, or polyols serve as such heat protectors in yeast.

Klibanov (866) recently reviewed factors that stabilize enzymes against thermal inactivation. There are a number of reports in the literature on stabilization of enzymes by polyols (867). From their studies in hyperthermia and cancer treatment, Henle and co-workers (865) recently proposed a model whereby cells can increase their thermal resistance based on the production of polyhydroxy compounds - polyols, carbohydrates and their derivatives. Polyhydroxy compounds protect catalytic proteins against heat denaturation; presumably by the interaction of water with the hydrophobic interior of globular proteins (868). Sugars (glucose, mannose, and especially galactose) and polyols also have been reported to protect whole cells against death (869, 870). In fact thermotolerant and polyol protected cells show similar heat survival curves under various environmental and treatment conditions (870). Although cells under short term nutrient deprivation were protected by glycerol, cells under prolonged nutrient deprivation conditions, were more sensitive to hyperthermia by glycerol (870). This implied that glycerol protection required an unknown nutritional factor or a vital protein. Paris and Pringle (871) recently reported that starved cells of *S. cerevisiae* were more heat sensitive than normal cells. They raised the question of whether thermal resistance was a general characteristics of non-proliferating cells.

Polyols may play a role in the osmotic stability of osmophilic yeasts, yeasts which are capable of growing in high solute concentrations and low water activity (872). Osmophilic yeasts are able to grow on utilizable carbon sources like glucose at 40°C, only in a saline medium (872, 873). The basis for this is presently unclear. Temperature resistance of osmophilic yeasts is also increased in concentrated media (874). The role of polyols in thermotolerance of yeasts is an area which deserves examination.

The presence of sugar in the medium can often have a detrimental effect on yeasts. Wells (875) reported in 1917 that the presence of sugars raised the thermal death point of *S. cerevisiae*. Van Uden and Madeira-Lopes (876) reported that the critical glucose concentration for growth of *S. cerevisiae* increased with the cultivation temperature. Edgley and Brown (877) recently examined the physiological changes induced by solute stress in yeasts. *S. uvarum (carlsbergensis)* in water suspensions could readily survive temperatures of 40°C; however, addition of glucose either simultaneously or sequentially, caused substantial irreversible leakage of cell contents (878). This was found not to be due to the osmotic pressure of the medium but rather to the specific presence of fermentable sugar (566). Sugar transport may be involved since materials that inhibited sugar uptake decreased leakage (566, 879). The rate of leakage and damage increased with temperature until the T_{max}; thereafter, there was no further leakage. In the absence of glucose, there was no leakage observed even above the T_{max} (566).

The foregoing may explain in part the discrepancies between maximal temperatures for growth versus survival of yeasts. Many tests for thermal injury have previously been conducted in water, whereas growth has been measured in media containing utilizable sugars. Although the presence of fermentable sugar at high temperatures induces damage and inhibits growth, it might well lead to enhanced fermentation. It was shown that yeasts exposed to elevated temperatures produce twice as much CO_2 in the presence of added glucose than in its absence (in heated water) (880).

In preliminary studies, calcium was found to protect cells against this glucose-induced leakage suggesting an effect on cytoplasmic membranes (878). This fact has subsequently been confirmed (566, 846, 881). Many investigators believe that it is the combination of high temperature and of fermentable sugar that results in membrane damage, and hence they believe that both factors are responsible for controlling the T_{max} (165, 566, 878). The effects on T_{min} on the other hand seem to be related to enzyme activity and substrate transport (174).

Since the rate of yeast fermentation can be affected by various physiological factors [ammonium ion, medium composition, redox balance, inoculum level or the stage in the growth curve (559, 560, 882-886)] it follows that fermentation would also be affected by these factors at higher temperatures. Jones and Greenfield (887) recently published a review on the role of ionic nutrition in yeast growth and in production of fermentation ethanol. As a consequence of a range of chelating agents in industrial media (leading to trace element imbalance) it is difficult to assess the necessary ionic concentrations at high temperatures. Unfortunately, there have been very few studies conducted on the effect of physiological factors on fermentation of yeasts at high temperature. Loginova (820) in 1962 wrote a book entitled "The Physiology of Experimentally Obtained Thermophilic Yeasts". Although it reviews factors affecting growth and fermentation of yeasts at high temperatures, and effects of high temperatures on yeast cells, it has yet to be translated from its original Russian version.

Unsaturated fatty acids such as linoleic or oleic are known to increase membrane fluidity in yeasts (888, 889). The steric hindrance of the double bonds in the side chains of unsaturated fatty acids results in a lipid bilayer that is very fluid even at low temperatures (192). The increase in saturated fatty acids at higher temperatures may provide the correct degree of rigidity required for membrane function under these conditions. However, it may also be responsible for the greater toxicity of ethanol observed at high temperatures. The more rigid membrane caused by the greater amount of saturated fatty acids at higher temperature, may prevent intracellular ethanol, the truly toxic metabolite, from leaving the cell (Section IX, 2b).

It has long been recognized that yeasts require oxygen in order to synthesize unsaturated fatty acids and sterols for growth (822, 890). In fact, for anaerobic growth of yeast, both oleic acid and ergosterol must be added to the medium (890). Since higher temperatures lower oxygen solubility, it is possible that yeasts require a greater amount of exogenous fatty acids and sterols to grow at elevated temperatures. Sherman (829, 857) reported that growth of *S. cerevisiae* at 40°C resulted in an increased nutritional requirement which could be met by supplementation with ~8% yeast extract or oleic acid (1 μmole/ml). This nutrient requirement could be eliminated by adapting the cells to growth at 40°C. In contrast, the optimal growth rate of *S. cerevisiae* at 30°C was independent of the concentration of yeast extract above 0.5% (w/v).

Sterols, polycyclic lipid-alcohols are found in yeasts, but generally are absent in procaryotes (891). They are thought to be an important component of eucaryotic cellular membranes and are synthesized and present in large quantities in the mitochondria (892). Recent results suggest that there may be two different domains in the yeast plasma membrane - sterol-rich and sterol-poor (893). The biosynthesis of sterols in yeasts (reviewed in 894) is affected by many physiological factors including temperature. Aerated yeasts synthesize less sterols at high temperatures partially due to an inhibition at elevated temperatures of the enzyme activity involved in ergosterol synthesis (895). Starr and Parks (896) showed that addition of ergosterol at 40°C stimulated growth and alleviated the requirement of yeast extract at elevated temperatures. Although the precise function for sterols in yeasts has not yet been elucidated, a large body of evidence suggests that sterols may play a role in the development and maintenance of respiratory competency (894-898). In addition, evidence (Section IX, 2b) suggests that sterols are important for enhanced ethanol tolerance and/or production in yeast. It is also noteworthy that both elevated temperatures and ethanol induce respiratory-deficient or petite mutants, and it has been suggested that the inner mitochondrial membrane is the target site for ethanol enhancement of thermal death (Section IX, 2c). Perhaps this may explain the greater requirement for ergosterol at elevated temperatures (895, 896).

Since ethanol toxicity to yeasts is accentuated at high temperatures, it seems quite possible that supplementation of the media with fatty acids or sterols might increase ethanol production at elevated temperatures. A few recent studies have shown this to be the case. *S. uvarum inulyticus var. nov.*, a new ethanol tolerant yeast strain from rice wine, produced up to 9.8% v/v at 40°C within 36-48 hours, from a step-wise addition of sucrose (596). The addition of fungal mycelia extract into the synthetic medium increased the fermentation rate and ethanol yield up to 11.6% v/v. Another study examined the fermentation ability of two *S. cerevisiae* strains (1200 and NP-3), on molasses media at 40°C with and without supplementation (590). As seen in Table 35, supplementation of the molasses medium with either lipid-protein complex or grated carrot increased the ethanol yield. These workers concluded that the lipid-protein complex, a high concentration ethanol-producing factor (425, 899), was less effective on each strain than grated carrot. However, it should be noted that in their fermentation experiment with the lipid-protein complex, a smaller inoculum of cells was employed than in the experiment utilizing grated carrots (1x10^8 vs 3x10^8/ml). Despite obtaining very high concentrations of ethanol at 40°C (up to 13% v/v), there was still a significant decrease in ethanol concentration as compared to 30°C (up to 22% v/v). However, at the higher temperature the fermentation rates were significantly improved; fermentation time was 40-80 hours as opposed to 6-10 days at 30°C. The factors contained in the grated carrot promoting alcohol tolerance to yeast cells at high temperatures are presently unknown. It was previously reported that the lipid-protein complex isolated from *Aspergillus oryzae* promoted growth, enhanced fermentative ability, and ethanol durability in yeasts (425, 899-901). The essential component for the high concentration alcohol-producing factor was a complex of unsaturated fatty acid-containing phospholipids (phosphatidylcholine) with macromolecules (899, 902).

Saigal and Viswanathan (903) found that the addition of vegetable oils and particularly of the fatty acids derived from them, stimulated growth, and increased both thermotolerance and ethanol tolerance of *S. cerevisiae*. This was demonstrated by a higher viable cell count after 72 hours from cells incubated at 40-45°C in the presence of 0.5% (v/v) linseed oil and 5-10% (w/v) ethanol. In contrast to the report of Rudenok and Konev (862), similar results were obtained with initial low and high inoculum values. Investigators from the National Sugar Institute in Kanpur, India, also observed that 0.5% (v/v) linseed oil, cotton seed oil, or vegetable oil and the fatty acids derived from them, stimulated molasses fermentation by a distillers yeast at 30 and 40°C (904). At 30°C, supplementation with linseed oil and especially fatty acids derived from it, significantly improved fermentation efficiency (Table 36). The rate of sugar utilization and final ethanol yield were lower at 40°C in the control than at 30°C. However, at this temperature, supplementation of the medium with linseed oil, or of fatty acids derived from it, improved the rate of sugar utilization, and ethanol production, resulting in a higher fermentation efficiency and ethanol yield (Table 36).

Saigal and Viswanathan (903, 904) concluded that the supplemented oils and fatty acids altered the membrane composition and structure in a favorable manner, thereby reducing the harmful effects of higher temperature. It should also be pointed out that not all oils will protect cells at higher temperatures. Mustard oil gave lower sugar utilization and ethanol production values than the control, probably as a result of some inherent inhibitory substances (903, 904). Furthermore, it has been reported that essential oils of plants (i.e., spices) prohibit repair of thermal injury in yeasts (905).

The precise mechanism(s) involved in membrane stability remains evasive. Changes in the physical state of the membrane induced by changes in fatty acid composition, probably represents a necessary but certainly not a sufficient condition, for the existence of life in thermal environments (193). It is also possible that the rigid membrane caused by the saturated fatty acids is just incidental and not necessary for survival at high temperatures. Brock

(33) has suggested that the inability of eucaryotes to grow at high temperatures may be due to an inability to form thermostable and functional organellar membranes. Since these intracellular organellar membranes must allow the selective passage of large macromolecular components they must have large pores and be relatively leaky. Conceivably, these leaky or permeable membranes are not compatible with thermostability.

Membrane rigidity may be required for the function of certain membrane-bound enzymes or it may be required to prevent dehydration. Womersley (906) recently reviewed the biochemical and physiological aspects of dehydration. It remains to be determined whether water loss is accentuated at higher temperature. This could be determined by measuring the water activity of yeasts at a series of temperatures by a variety of techniques (hygrometer, thermocouple psychrometer, nuclear magnetic resonance, etc.) (907-909). It has been reported that bakers yeast looses water due to evaporation during storage (910).

It has been speculated that the key to survival of a number of organisms in the absence of water could be the production of the reserve carbohydrate, trehalose (906, 911). Crowe (911) recently postulated that an interaction between trehalose and phospholipids in cell membranes may prevent damage from dehydration by direct inhibition of phase transitions in membranes. In fact, glycerol and trehalose may interact with lipid membranes and reduce the amount of leakage (906, 912). It has been reported that the large pools of trehalose present in bakers yeast remain relatively constant, if the cells are starved by incubation in water (913). It is also noteworthy that trehalose often accumulated transitorily under adverse conditions (eg., exhaustion of nutrients, stationary phase of growth, germination) (914-916). The importance of trehalose in growth and regeneration of yeasts has already been implicated (917). Other studies have shown that the trehalose content of *S. cerevisiae* decreased during dehydration and lyophilization, but was restored upon rehydration (918, 919). Grba *et al.* (920) reported that bakers yeast synthesized trehalose during the exponential growth phase at 37°C, but not at 30°C. One study reported that a strain of *S. cerevisiae* that was more "dry tolerant and thermotolerant" may have contained more trehalose in its cells (921). Recently, Keller *et al.* (922) postulated that trehalose may function as a protective agent to maintain the cytoplasmic structure under conditions of stress. In addition, several strains of yeast are able to ferment extracellular trehalose due to its hydrolysis to glucose by the presence of trehalase (923).

In view of the fragmented pieces of evidence, one may postulate the following hypothesis. Thermotolerant microorganisms may produce more trehalose at high temperatures as a survival mechanism to prevent dehydration. Perhaps, physiological or genetic changes which increase trehalose accumulation in microbial cells may permit survival at higher temperatures without the requirement for a rigid membrane. If so, the addition of unsaturated fatty acids to this trehalose enriched organism, may allow for better fermentation at high temperatures. Hypothetically, the unsaturated fatty acids would provide a more permeable membrane which could allow intracellular ethanol to leave the cell, and the accumulated trehalose would protect cells from desiccation.

c. Heat Shock Proteins

Another factor that may affect yeast growth and/or fermentation at high temperature is the presence of heat shock proteins (hsp). Heat shock, a brief exposure of cells to high temperatures (usually 37°C for 30 minutes), induces a set of specific hsp's (usually $\leq$16) which confer resistance of the cells to high temperature. The heat shock response appears to be universal and was first described by Ritossa (924) in *Drosophila*, an organism commonly used in investigating the regulation of gene activity associated with heat shock. In *Drosophila* the major heat shock induced protein is called hsp 70 (925). It is also manifested at the cellular level in bacteria, yeasts and other eucaryotes. In bacteria (926, 927), these proteins are sometimes referred to as high temperature production proteins (htp) (928). The coincident appearance of acquired thermotolerance and hsp's has led to the idea that hsp's may represent an adaptation mechanism for survival at high temperatures. However, the mode of action of these proteins remains unclear.

Besides heat, a variety of other cellular stressors such as amino acid analogs (929), metal ions such as Hg^{+2}, Cd^{+2}, and Zn^{+2} (930), cellular trauma (931), UV light (932), arsenite (925, 933), oxygen deprivation (925), and ethanol (933-936), can induce synthesis of hsp's. These findings suggest that hsp's may play a role in a generalized cellular adaptation to stresses such as incubation at high temperature, exposure to ethanol, or sporulation. Bilinski and Miller (937, 938) demonstrated that either of two treatments known to induce synthesis of hsp's, heat shock or zinc, can suppress expression of certain recessive mutations affecting sporulation in *S. cerevisiae*. In fact it has recently been shown that the nutritional manipulation which suppressed the mutations, induced synthesis of hsp's (Bilinski - personal communication). Therefore, it is conceivable that thermotolerance as an adaptation to stress, can be acquired by nutritional manipulation. In this regard it is noteworthy that zinc, as well as other minerals, offers some protection against heat (Section IX, 1d). Minton and co-workers (939) recently proposed a model whereby hsp's could contribute to enhanced stress tolerance in cells by nonspecific stabilization of stress-susceptible proteins by stress-resistant proteins. This hypothesis should be tested using actual hsp's rather than stress-resistant proteins; however, such experiments would require the purification of large quantities of hsp's.

Li *et al.* (933, 934, 940, 941) found that ethanol induced the synthesis of hsp's and confered thermotolerance in Chinese Hamster fibroblasts. Similarly preincubation of yeast in 5-7% (w/v) ethanol, induced these hsp's and thermotolerance (935). However, it was noted that hsp's and thermotolerance in yeast were greater after heat shock than after ethanol preincubation. Watson and Cavicchioli (936) reported that heat shock of *S. cerevisiae* not only induced hsp's and thermotolerance, but also imparted some degree of ethanol tolerance. Li *et al.* (940) also reported that thermal pretreatment resulted in an increased ethanol tolerance in Chinese Hamster cells. It is noteworthy that both heat and ethanol induced petites and this will be discussed subsequently (Section IX, 2c). It would be of interest and importance to evaluate the fermentation ability of heat shocked yeasts at higher temperatures, especially in view of the fact that microorganisms demonstrate greater inhibition by ethanol at elevated temperatures.

Furthermore, if there is a set of hsp's associated with the acquisition of both ethanol tolerance and thermotolerance, this would offer the possibility of cloning these genes into microorganisms in order to express both desirable traits simultaneously.

Finkelstein and Strausberg (942) cloned a gene for an abundantly synthesized hsp into yeast (representing up to 1/5 of the protein synthesized *de novo*). No effect on synthesis of other major hsp's was observed and no alternations were noted in the phenotypic response of heat shock acquisition of thermotolerance. However, this finding does not preclude the possibility of cloning regulatory genes and other components which might be involved in the acquisition of ethanol tolerance or thermotolerance in yeasts. In this regard, evidence suggests that regulatory genes controlling hsp's exist (928, 943, 944).

S. cerevisiae contains a multigene family related to the *Drosophila* heat shock gene *hsp* 70, and two of these yeast genes contained on plasmids *YG*100 and *YG*101 have been characterized (945). The yeast *hsp* 70 genes were truncated and fused to the *E. coli lac* Z gene (946). This permitted heat shock gene expression to be easily detected by a sensitive assay system (946). Although the expression of the *YG*101 gene could not be induced by any stimuli, *YG*100 gene expression could be induced over 20-fold by an increase in temperature (944). Expression of the heat shock gene was also induced at late growth phase and high cell densities, physiological conditions where yeasts are more thermotolerant (944). One explanation to account for this is that cells induce heat shock gene expression as nutrients are depleted

(944). Expression driven by the *YG*100 gene could be induced to over 50-fold on recovery from anoxia - the reintroduction of oxygen after a prolonged incubation under anoxic conditions. The thermotolerance of yeast after recovery from anoxia is an area that should be studied further. Many physical and chemical stimuli tested that were previously reported to induce hsp's or generally stress cells, (UV irradiation, dimethyl sulfoxide, sorbitol, zymolyase treatment, ethanol), did not stimulate gene expression (944). However, it is possible that *YG*100 is not a member of the group of heat shock genes that are induced by these stimuli. Therefore analysis of the response of other heat shock genes to various stimuli is necessary. Finkelstein and Strausberg (943) have shown that another gene, *hsp* 90, may also direct the heat shock controlled expression of β-galactosidase in *S. cerevisiae* with an appropriate fusion.

Heat shock alters the transcription of certain genes (947). During the heat shock response, the synthesis of many proteins (eg. ribonuclease) (948) are induced while others are repressed, and yet still others are unchanged (949). The consensus for some time has been that hsp's are not necessarily newly synthesized proteins, but rather constitutively expressed ones that are normally synthesized at a basal rate, and whose synthesis is greatly increased in response to stress (950). In most studies, the heat shock response consists of shifting the temperature from 23 to 37°C for 30 minutes, and observing the protection of cells at 52°C (951). The previous growth temperature and stage of growth of cells subjected to heat stress is also an important factor contributing to survival (936, 952). It has recently been reported that acquisition of thermotolerance in the fungus *Ustilago maydis* does not depend upon recombination (932). McAlister and Finkelstein (951) in 1980 concluded that hsp's were directly involved in thermotolerance. The addition of the protein synthesis inhibitor cycloheximide (100 µg/ml) prior to temperature shift, eliminated the acquisition of thermotolerance. Others have reported similar findings (927, 932, 941, 953).

A large body of evidence is available in the literature to support the conclusion that factors other than hsp's appear to be involved. Occasionally, the amount of protein synthesized in response to heat shock does not exceed that synthesized when the cells are continuously grown at 42°C (927). The thermotolerance is transient and it decreases after further growth (927, 950). Contrary to McAlister and Finkelstein (951), Hall (954) found that yeast cells (including McAlister's strain) acquired the thermotolerant state in the presence of cycloheximide (25-100 µg/ml). In similar experiments, Landry and Chretien (955) showed that hsp synthesis was not a sufficient condition for thermal resistance in Morris Hepatoma cells. In view of the importance of these controversial results, Watson *et al.* (956) examined the effect of cycloheximide (100 µg/ml) and chloramphenicol (4 mg/ml) on thermotolerance in 4 strains of yeast in over 30 separate trials. Both cytoplasmic and mitochondrial protein synthesis were found not to be required for the acquisition of thermal or ethanol tolerance in yeast. Recent hyperthermia studies in Chinese Hamster Ovary (CHO) cells, implicated ADP-ribosylation in the acquisition of thermotolerance (957).

It has recently been suggested that the acquisition of thermotolerance is dependent on the stage in the cell division cycle attained by cells at the time of heat shock, rather than on induction of hsp's (958). Cell fractions enriched with small unbudded cells or cell cycle mutants arrested at G1 were found to be more thermotolerant than cells from other cell cycle stages (958). Vanoni *et al.* (959) recently analyzed the effects of temperature on the yeast cell cycle by flow cytometry. A temperature compensatory mechanism appears to operate in the S phase, while duration of G_2 + M phases, exhibit a much more pronounced temperature dependence. It is interesting to note that there is a cell cycle dependence of heat sensitivity and thermotolerance development in CHO cells (960).

It is possible that thermotolerance is caused by the activity of a pre-existing component which is activated at 37°C or by other factors. In a similar manner, a pre-existing component which interferes with thermotolerance may be inactivated at this temperature (961). This activation or inactivation might possibly result in the coincidental production of hsp's which have little function for thermotolerance. It is also possible that the levels of cycloheximide used by investigators (951, 955, 956) were not sufficient to inhibit total protein synthesis. Recently, the effects of cycloheximide on the temperature profile of *S. cerevisiae* was examined (962). In fact, Landry and Chretien (955) reported that only 90% of protein synthesis was inhibited in their experiments with cycloheximide. It may be that the few proteins induced are sufficient to elicit a full-thermotolerant response. It is clear that more research is needed in this area and this might eventually lead to enhanced resistance to high temperature and/or ethanol.

2. Ethanol Tolerance

a. Bacteria

One of the major limitations in the conversion of saccharides to ethanol by thermophilic ethanologenic bacteria is their marked intolerance to ethanol. Thermophilic bacteria are also inhibited by their other products, especially acetic and lactic acid. Efforts however, have focused mainly on obtaining ethanol tolerant mutants. This is due in part to the finding that ethanol tolerant isolates usually produce less acetic and lactic acid as by-products (439). However, it should be noted that an adaptation to ethanol will not necessarily result in an adaptation to the other products. This can only be achieved by increasing the concentration of each product either simultaneously or individually in small increments.

As a consequence of inhibition by ~0.5-1% (w/v) ethanol, thermophilic ethanolgenic bacteria do not efficiently ferment substrate concentrations greater than 1-2%. In view of the numerous potential advantages of these bacteria, various research programs have been directed towards improvement of their ethanol tolerance. In fact, as will be discussed, it can be seen that higher ethanol producing strains have resulted through various mutation, selection, and adaptation schemes. Although the specific techniques vary between laboratories, the general procedure for the selection of ethanol tolerant bacteria is similar to the MIT adaptation selection procedure outlined (439, 442, 693). The bacteria are cultured in a medium containing a suitable carbon source, such as cellobiose or xylose, but in addition, ethanol

(~1% w/v) is often included. Cultures are then serially transferred to progressively increasing ethanol concentrations at increments of ~0.2-0.5% (w/v). More resistant isolates can often be obtained by subculturing the microorganism twice at the same ethanol concentration (11). Frequently, the cultures are mutagenized with ethyl methane sulfonate or nitrosoguanadine, and selection is for reduced lactate and/or acetate producers, since ethanol tolerant isolates produce less of these acids. This is accomplished with the aid of various dyes (tetrazolium or methyl red) or by enzymatic reactions (369, 439, 442, 713). For example, in the presence of methyl red, acetic acid producing colonies will be brighter (442). Other strategies involve selection for fluropyruvate resistant mutants, pyruvate negative mutants, or by modifications in the iron content of the medium (59, 369, 467, 815).

This basic adaptation, mutation, and selection procedure employed at MIT has resulted in improved ethanol tolerant strains of *C. thermocellum* (S-4, S-6, and S-7) and *C. thermosaccharolyticum* (HG-3, HG-4, and HG-6) as discussed earlier (Section VII, 2c). Other researchers have selected ethanol tolerant mutants of these strains by similar schemes (427, 518, 713). Lovitt *et al.* (492) obtained an alcohol tolerant strain of *C. thermohydrosulfuricum* (39EA) (Figure 22). Studies showed that ethanol tolerance was temperature dependent in the mutant but not the parent. The mutant grew with ethanol concentrations up to 8% (w/v) at 45°C, 5% (w/v) at 60°C, and 3.3% (w/v) at 68°C. Ethanol tolerance in the mutant was attributed in part to a unique membrane lipid composition containing C_{30} dicarboxylic acids, proposed to be in the form of a tetraester of glycerol (492). Presumably, membranes containing this component would not be subject to solvent disruption by hydrophobic interactions between free fatty acid side chains present in normal lipids (492). Investigations have also produced other ethanol tolerant isolates of *C. thermohydrosulfuricum* (62, 541, 542, 705, 963).

Carreira and Ljungdahl (441) were also very successful in adapting *T. ethanolicus* to higher ethanol concentrations (up to 10% v/v). As discussed earlier (Section V, 1c), mutant *T. ethanolicus* JW200 Fe(4) produced up to 3% (w/v) ethanol from starch. It appears that this thermophilic ethanologen has the highest tolerance to ethanol, gives highest ethanol yields, and is the quickest to adapt to ethanol (441). In fact, the wild type bacterium will grow in the presence of 9% (w/v) ethanol, after a simple 3 step transfer from 3 to 6 and then to 9% (w/v) ethanol (Carreira - personal communication). In contrast, the mutant will tolerate 9% (w/v) ethanol without any adaptation. The stage of growth of the bacterium was found to play an important role in determining ethanol tolerance. Thermophilic bacteria were found to be more ethanol intolerant, when ethanol was added at the time of inoculation, rather than during the exponential growth phase (Carriera - personal communication).

Wild type *C. thermohydrosulfuricum* strains grew readily in >4% (w/v) methanol and acetone, but were sensitive to 2% (w/v) ethanol (492). Therefore, the basis for their ethanol intolerance was not due to solvent effects on membrane integrity as has been suggested for other organisms (431, 964-966). In fact both Zeikus' (492) and Wiegels' (59, 441) laboratories have attributed ethanol intolerance to a regulatory problem - an ethanol-dependent inhibition of ADH (Section IX, 1b).

Herrero, Gomez, and co-workers at MIT (707, 967-971) have studied the mechanism of ethanol tolerance in *C. thermocellum* ATCC 27405 and alcohol tolerant mutants C9 and C919. The growth of *C. thermocellum* is inhibited by n-alkanols, with the degree of inhibition increasing with the chain length (967). Whereas growth of the wild type on 0.5% (w/v) ethanol was inhibited by ~50%, the mutant *C. thermocellum* C9 required 2% (w/v) ethanol to exhibit a similar degree of inhibition (707).

Exponentially growing cells of *C. thermocellum* exposed to ethanol are characterized by three successive phases (707, 967): an initial period of uninhibited growth (phase I), a period of growth arrest (phase L), and a period of inhibited but sustained exponential growth (phase II). Phase I and L are dependent on ethanol concentration and other physiological conditions. For example, extended periods of growth arrest are observed with cells in the stationary growth phase. Both the wild type and the mutant adjust their fatty acid composition upon growth in ethanol to produce a phase II membrane, which has fatty acids with lower melting points. One of the most detectable alterations in membrane composition of *C. thermocellum* induced by ethanol is the increase in normal and anti-isobranched fatty acids at the expense of isobranched fatty acids (969).

The biological response to ethanol is to fluidize the membrane (333, 972). It has been proposed that the purpose of this response is to counteract the physical effect of ethanol on membrane permeability. On insertion of ethanol between membrane lipids, water molecules are displaced and hence the membrane tightens (333, 707). Consequently, this can alter a number of cellular functions which depend upon membrane fluidity (eg., lipid/protein interactions, membrane-protein mediated phenomena) (707). Herrero and Gomez (968) have confirmed the existence of homeoviscous adaptation in *C. thermocellum* in response to ethanol. However, it has been reported that the alcohol tolerant mutant does not change its fatty acid composition as significantly as the wild type on the addition of ethanol (969). It was hypothesized that the mutant was defective in a "fluidity sensor or effector" as it was not able to detect and counteract changes in membrane fluidity (969). It was concluded that fatty acid changes on adaptation of the organism to ethanol were not necessarily direct responses to alter membrane fluidity, but instead were most likely secondary responses. Ethanol may alter substrate specificity of enzymes and therefore cause random changes in protein/membrane interactions (973). It is possible that the ethanol tolerant mutant has altered its fatty acid producing systems.

The phase behaviour of the membrane lipids from the wild type and ethanol tolerant mutant *C. thermocellum* C919 were examined by differential scanning calorimetry (970). The lipids from the parent demonstrated a broad phase transition (~30°C), while the lipids from the mutant exhibited a 10°C lower transition temperature. When adapted to growth in ethanol, the membrane lipids from the wild type exhibited altered phase behavior and a broadened and lower T_m (15-25°C). In contrast, the lipids from the ethanol tolerant mutant did not show significantly altered phase behavior when adapted to growth in ethanol (970).

Kundu *et al.* (974) recently attributed the inhibitory effects of ethanol on *C. thermocellum* to be due to the combined effects on cell viability, enzyme systems, and the membrane composition of the cells. Herrero (971) recently conducted studies to determine the target(s) which controls the rate of growth when *C. thermocellum* is affected by ethanol. The presence of ethanol does not affect the ΔpH (i.e., cause protons to leak through the membrane), as does acetic acid, nor does it force the cells to use more ATP. This is in contrast to the recent findings that show ethanol acting as an uncoupler in yeasts (975). Studies dealing with the kinetics of ethanol inhibition exhibit a close relationship between growth retardation and a reduction in the rate of sugar utilization (707, 976). Since the level of growth inhibition correlates well with an increase in cell content of sugar phosphates, this rules out an inhibition in sugar transport (971) as was seen with yeasts (558, 889, 977). Some reports indicate that growth inhibition of yeasts may be a direct result of the irreversible denaturation of some of their glycolytic enzymes such as glucokinase (Section IX, 2b). Although the particular sugar phosphate that accumulates has not been unequivocally identified, it is thought that the target enzyme is located before the steps yielding glyceraldehyde 3-phosphate (971). Since some glycolytic enzymes are membrane bound (978), it is possible that the change in lipids, and hence membrane fluidity in ethanol inhibited cultures, may result in less active glycolytic enzymes, and therefore, growth inhibition.

As with *S. cerevisiae* (979) and *C. thermocellum* (968), many bacteria such as *Bacillus spp.* and *E. coli* have been shown to alter their lipid composition during growth in the presence of ethanol (333, 431, 980-983). A change in sensitivity to ethanol may be caused by a change in the proportion of different phospholipid classes (984). The proportion of acidic phospholipids such as phosphatidylglycerol and cardiolipin increases as does vaccenic acid (C_{18}), whereas the amount of phosphatidylethanolamine decreases (981). It is noteworthy that *B. stearothermophilus* has a higher concentration of cardiolipin compared with other mesophilic species (273, 985). Furthermore, the steady state concentration of cardiolipin increased with growth temperature and upon anaerobic conditions (273). The ethanol-induced changes in lipid composition of *E. coli* are proposed to be beneficial for growth and tolerance in the presence of ethanol (986, 987). Mutants of *E. coli* that are unable to synthesize vaccenic acid are hypersensitive to ethanol (986) while ethanol-resistant mutants contain higher amounts of acidic phospholipids (984). The enzymology, genetics, and regulation of membrane-phospholipid synthesis in *E. coli* has been previously reviewed (988). Recent results of Dombek and Ingram (987) suggest that the regulation of the lipid-to-protein ratio of the plasma membrane, may be an important adaptive response of *E. coli* to growth in the presence of ethanol.

The fatty acid composition of *Z. mobilis*, an ethanol producing ethanol tolerant microorganism (Section IV, 3), represents an extreme of the trend observed in ethanol-induced *E. coli* (417). In *Z. mobilis*, vaccenic acid represents ~78% of the acyl chains in polar membrane lipid fractions, and phosphatidylethanolamine is the dominant phospholipid. Unlike most other organisms, the fatty acid composition does not significantly change as a result of ethanol additions (432). However, ethanol induces other changes in membrane composition of *Z. mobilis*: shifts in phospholipid composition (eg., increase in cardiolipin and phosphatidylcholine) and a decrease in the lipid-to-protein ratio. It has been suggested that the fatty acid composition of *Z. mobilis* may represent an evolutionary adaptation for survival in the presence of ethanol possibly due to its metabolic capability i.e., high fermentation ability as compared to oxidative metabolism (432). However high levels of vaccenic acid have been found in other non-ethanol producing bacteria (eg., *Rhodopseudomonas sphaeroides*) as well (989).

On the basis of studies with *Z. mobilis*, *E. coli*, yeasts, and other microorganisms, a clear trend is emerging in the relationship between ethanol tolerance and fatty acid composition, i.e., longer-chain unsaturated fatty acids appear beneficial (432). Long chain saturated fatty acids which are beneficial for survival at higher temperatures (Section III, 3), are not produced in the presence of ethanol and may be harmful (432).

It was also found that temperatures in excess of 30°C increase the sensitivity of cells to ethanol inhibition, and ethanol lowers the thermotolerance of *Z. mobilis* by acting on the cell membrane (417, 429-431). Similarly, high concentrations of glucose (high osmotic pressure) increase the sensitivity of the cells to inhibition by ethanol (417, 432). Since high initial sugar concentrations, ethanol, or temperatures also reduce fermentation efficiency, it has been suggested that the cell membrane may be the common site of action for the synergistic effects of ethanol, thermal, and osmotic stress on *Z. mobilis* (417).

Unique lipids may also be responsible for ethanol tolerance in *Lactobacillus heterohiochii* and *Lactobacillus homohiochii* (990-993), the most ethanol tolerant microorganisms known to date ($\geq$20% w/v ethanol) (417, 431). These bacteria produce phospholipids rich in 20-to-24 carbon mono-unsaturated and cyclopropane fatty acids (991, 992). It may be that the longer chain unsaturated fatty acids increase the thickness of the cell membrane and thus offer protection (431). In addition, unlike most other bacteria which produce saturated fatty acids at high temperature (Section III, 3), *L. heterohiochii* continues to synthesize unsaturated fatty acids as the growth temperature is increased (992). Unfortunately *L. heterohiochii* is incapable of ethanol production (417).

Gene banks of *L. heterohiochii* have recently been screened for genes coding for ethanol tolerance in *E. coli* (417). The genes for the enzymes responsible for synthesizing unique lipids in *L. heterohiochii* (suspected enzymes which control fatty acid elongation) have been cloned and expressed in *E. coli* (Ingram - personal communication). Since introduction of this property into *E. coli* has imparted some ethanol tolerance, efforts are currently in progress to clone this gene into *Z. mobilis*. However, insertion of genes into *Z. mobilis* is a long term project incorporating development of suitable vector systems for gene insertion and maintenance, and improvement of gene expression (promoters, excretion sequences) (417, 994, 995). The lactose operon from *E. coli* which has been inserted into *Z. mobilis* and is expressed, serves as a model system to investigate the expression of foreign genes (996).

b. Yeasts

Ethanol intolerance of yeasts is a fundamental problem in the fermentation industry. This section will briefly review the adverse effects of ethanol on yeast. Other reviews on the effects of ethanol on yeasts have been published elsewhere (431, 965, 997, 998).

As early as 1928, Richards (999) demonstrated that alcohol inhibits yeast growth. Much of the interest on the physiological basis of ethanol tolerance in yeasts has arisen from Gray (558, 1000-1002) and Troyers' (1003, 1004) work in the 1940's and 1950's. It was reported that ethanol tolerance was not limited to any one genus or species of yeast and that yeasts were less ethanol tolerant at higher temperatures (558). Gray (1001) also found that ethanol tolerant yeasts stored less lipid and carbohydrate material than less tolerant strains. Although yeasts exhibiting high glucose tolerance also exhibited good ethanol tolerance, glucose acclimatized strains showed lower ethanol tolerance (1000). This may be due to a "glucose tolerance factor", which increases glucose tolerance and the decarboxylation of pyruvate to ethanol (1005). Troyer (1003, 1004) confirmed most of Gray's findings; in addition, he concluded that ethanol intolerance was not due to the fact that ethanol was a yeast metabolite, since methanol also inhibited sugar utilization in *S. cerevisiae* (1004). It was also shown that toxicity due to exogenous ethanol, was not due to impurities present in the ethanol (1006). The inhibiting effect of alcohols increases with the carbon number; however, secondary alcohols are less inhibitory than their respective primary isomers (1002). After Gray and Troyers' reports, the subject of ethanol tolerance received little attention until 1974 when Hayashida and co-workers (425) reported that yeasts acquired enhanced ethanol tolerance when grown in the presence of a fungal mycelium extract containing unsaturated fatty acids. Since that time, this subject has received constant attention.

One of the fundamental difficulties in assessing ethanol tolerance in various yeast strains is that there is no universally accepted technique of measurement for ethanol tolerance. Ethanol tolerance has been measured by detecting growth on glucose in the presence of exogenous ethanol, and also by quantitating the amount of ethanol resulting from sugar fermentation. Frequently, when ethanol tolerance is measured, other physiological factors which can alter a cells susceptibility to ethanol are ignored (Table 1) (560, 1007-1010). Recently, Casey *et al.* (1011, 1012) reported that the factor limiting high yields of ethanol by brewing yeasts is a nutritional deficiency rather than ethanol toxicity.

To date, there is still disagreement as to the growth inhibiting effect of ethanol and whether this is due to accumulation of toxic levels within the yeast cell. In 1974, Nagodawithana *et al.* (1013, 1014) reported that exogenous ethanol, added to *S. cerevisiae* cultures, was less toxic than endogenous ethanol produced by the yeasts. This finding, confirmed by others (889, 972, 1015-1020), was thought to be due to the rate of ethanol production within the cells exceeding the rate at which it could be excreted under certain conditions (eg., rapid fermentations, high temperatures, high osmotic pressures) (1013, 1014, 1021). However, a lower exogenous ethanol toxicity could also be attributed to the slower permeability of the alcohol from extracellular to intracellular locations (1022).

There is also controversy surrounding the accuracy of the techniques employed to measure intracellular ethanol concentrations (972, 1019, 1023, 1024). Although details of methodology vary between laboratories, the general procedure involves the harvesting, washing, and disrupting of cells. Alternatively, undisrupted cells are sometimes injected directly into the gas chromatograph, and the intracellular ethanol concentration calculated from the knowledge of cell volume or water content (1016, 1022). Since the water content of the cell may change during fermentation, the assumption of a constant cell volume for calculation of the ethanol content of cells is questionable (972, 1023). Beavan *et al.* (972) also reported that washing yeast cells prior to measuring intracellular ethanol removed up to 96.2% of this alcohol. Since the cell membrane is highly permeable to ethanol, it seems unlikely that ethanol would bind to cell components and thus washing the cells was thought to be unnecessary (972). Hence, previous studies that employed cell washings prior to measurement of intracellular ethanol (1014, 1017) may have yielded conservative estimates of intracellular levels.

Dasari and co-workers (1023) recently suggested that intracellular ethanol concentrations previously reported were overestimates. The investigators pointed out that when the cells were harvested by centrifugation, any fermentation occurring after the cell sediment has formed tends to elevate the actual intracellular ethanol level because much of the ethanol produced would be retained in the sediment. Dasari *et al.* (1023) reported that the estimated ethanol concentration inside the cells significantly increased with centrifugation time. They also demonstrated the importance of precooling the sample before centrifugation to further reduce ethanol formation. It remains unclear whether there is any significant difference between extracellular and intracellular ethanol concentrations during the early part of fermentation (1023). In the latter stage of fermentation, Dasari and co-workers (1023) and

Goma *et al*. (1025) reported greater extracellular ethanol concentrations, suggesting the existence of an active transport system for ethanol efflux. However, many workers believe that movement of ethanol across the membrane is purely by diffusion, which is controlled by membrane fluidity and not by enzymes (767, 972, 1024).

Although there is still no accurate reported technique for measuring intracellular ethanol, Ingram claims to have developed a technique that measures the intracellular ethanol content of cells without washing the cells or breaking them open (Ingram - personal communication). In the latter stages of the fermentation, a higher concentration of extracellular ethanol (5-5.5% v/v) than intracellular ethanol (3.5-4% v/v) was found (1026). Similarly, resuspension of cells in fresh medium containing 10% (v/v) ethanol resulted in a significantly lower intracellular ethanol concentration of 7.5-8.5% (v/v) (1026). Guijarro and Lagunas (1024) recently reported on experiments involving the partitioning of radioactive ethanol, and suggested that intracellular accumulation of ethanol is not possible. Nevertheless, it is still believed in many laboratories that the ethanol concentration inside the cells can be greater than outside at certain stages of growth under special environmental conditions.

It is widely accepted that an increase in ethanol concentration adversely affects yeast viability (888, 1009, 1015, 1027) and the specific rate of yeast growth (889, 972, 976, 1003, 1009, 1028) and fermentation (888, 976, 1009, 1015, 1028). Table 37 lists other possible detrimental effects of ethanol on microbial cells. Many studies have examined the inhibitory effects of ethanol on yeast fermentation kinetics (560, 976, 1018, 1020, 1029, 1030). Ethanol inhibition of growth and fermentation is non-competitive (1031). Since ethanol inhibition varies with the initial substrate concentration, Mota *et al.* (1020) recently defined a new parameter to quantitate ethanol inhibition; "the added ethanol concentration at which biomass productivity declines to one-half its value with no added ethanol". Although the actual lethal mode(s) of action of ethanol is still unknown, there are many possibilities. The mechanism of ethanol-induced petites and enhancement of thermal death are discussed in the next section. Day and co-workers (1009) suggested that the viability of yeast cells was dependent upon the development of mitochondria able to oxidize ethanol via their ADH's. However, others have concluded that this is highly unlikely (888). Nagodawithana and Steinkraus (1021) observed that high intracellular ethanol concentrations during rapid fermentation are accompanied by inactivation of ADH and loss of cell viability. ADH's from *S. cerevisiae* have been isolated, characterized, and their regulation has been studied (1032-1034). Three different isozymes of ADH have been described in *S. cerevisiae*; two cytoplasmic enzymes (ADH I and ADH II) and one mitochondrial enzyme, ADH III. ADH I is responsible for ethanol formation, while ADH II and III are responsible for ethanol metabolism. Examination of ethanol tolerance and/or productivity of ADH regulatory mutants of *S. cerevisiae* (1035-1037), would be useful.

It was previously thought that ethanol replacement of water molecules associated with lipolytic enzymes could lead to inhibition of transport processes (333, 889, 983). Ethanol could modify the lipid content of transport proteins leading to conformational changes or it may permit lipolytic enzymes to hydrolyze phospholipids (1038). Van Uden and co-workers (977, 1031, 1039, 1040) have studied the kinetics of alcohol inhibition of glucose, maltose, ammonium, and amino acid transport across the membrane of *S. cerevisiae*. Ethanol mainly inhibits the transport of glucose and maltose at low alcohol concentrations, while at higher levels, ammonium and amino acids are more strongly inhibited (1031). The inhibition of nutrient transport is non-competitive and the effects of the alkanols on the maximum rate follow exponential kinetics (1031). Since the inhibitory potency of the alkanols increases with their lipid solubility, it was suggested that the alkanols inhibit nutrient transport by interfering with the transport mechanism in hydrophobic regions of the cytoplasmic membrane (1031).

It has been postulated that intracellular ethanol may be compartmentalized by being concentrated in a vacuole type structure (975, 1019). Although little research has been conducted in this area, there is no evidence to support compartmentalization (975). Hence it appears that all intracellular enzymes are bathed in high ethanol concentrations. Thomas and co-workers (888) hypothesized that death of *S. cerevisiae* resulted from the denaturation of intracellular enzymes following the passage of ethanol through the cell membrane. Since ethanol is known to be a protein denaturing agent (1041), it is likely that intracellular enzymes from ethanol tolerant yeasts are more resistant to the denaturing action of ethanol (975). There have been a few reports on the inhibitory effect of ethanol on yeast enzymes, and these are mostly the glycolytic enzymes, eg., glucokinase, α-glycerophosphate dehydrogenase (1042, 1043). Nagodawithana *et al.* (1042) suggested that ethanol behaved in a non-competitive manner to inhibit hexokinase by the formation of a reversible enzyme inhibition complex. This interaction may serve as a biofeedback system for ethanol production (1042). More recently, it has been concluded that enzyme denaturation is unlikely to play a major role in ethanol tolerance, especially below 10-12% (w/v), the level when fermentation normally ceases (1043). However, Millar and co-workers (1043) did agree that enzyme inhibition by ethanol, could be responsible for a decline in rates of fermentation as ethanol concentration increases. As discussed in the preceeding section, recent evidence in *C. thermocellum*, suggests that enzyme denaturation by ethanol may be the lethal target on the cell.

There is a body of evidence implicating the cytoplasmic membrane as the prime target site for the inhibitory effect ethanol has on the yeast cell (1044-1047). Recent findings suggest that ethanol may act as an uncoupling agent, disrupting the proton gradient across the membrane, and thereby causing an accelerated entry of protons into the cell (975, 1047). Rose (975) has presented preliminary evidence correlating the increase in ethanol tolerance of *S. cerevisiae* during exponential growth with the decreased ability of ethanol to uncouple the membrane.

There is considerable evidence indicating that unsaturated fatty acids and sterols are important for enhanced ethanol tolerance and/or production in yeast (425, 888, 899, 1011, 1012, 1048-1050). It has recently been shown that unsaturated fatty acids are more essential than sterols for high ethanol production in *Saccharomyces spp.* (902, 979). However, ergosterols synergistically stimulated the effect of unsaturated fatty acids on fermentation activity (901). The extra double bond provided by the side chains of the unsaturated fatty acids may provide additional stabilization resulting in a more effective barrier to the entry of ethanol into the cell (888). However, it appears more likely that the more fluid membrane resulting from the steric hinderance of this extra double bond, may facilitate excretion of intracellular ethanol.

Panchal and Stewart (767, 1017, 1051) reported that higher osmotic pressures resulting from greater solute concentrations, retarded the excretion of ethanol from the yeast cell. This resulted in a reduction in cell viability and fermentation rate. This may explain why sugar infusion or slow substrate feedings usually lead to enhanced ethanol production. This decrease in excretion of ethanol at higher osmotic pressures may be more problematic at higher temperatures, due to an even more rigid cell membrane. In this regard, Ziffer and Iosif (766) found decreased ethanol yields at elevated temperatures in high concentration corn mashes. When peptone or a surfactant was added to the yeast culture less ethanol accumulated (1016). In fact, it was shown that unsaturated lipids added to yeast cultures enhanced the excretion rate of ethanol from yeast cells (1052).

To date, no genes have been identified or characterized which confer high ethanol tolerance to any microorganism. For the reasons discussed, enrichment of unsaturated fatty acids may be important for enhancing ethanol tolerance. Examination of the biochemistry and genetics of lipid mutants may lead to a better understanding of ethanol tolerance (1053-1056). Temperature-sensitive mutants of *S. cerevisiae* defective in lipid biosynthesis have also been described (1057). Structural genes have been cloned for the biosynthesis of specific bacterial lipids (988, 1058). Such studies may provide a framework for the subsequent cloning of fatty acid genes that might enhance ethanol tolerance and/or productivity.

The complex nature of ethanol toxicity has led some researchers to conclude that it is presumably under genetic control (1009, 1059). Ismail and Ali (1060) reported in 1971 that ethanol tolerance in *S. cerevisiae* was controlled by a polygenic system and certain gene modifiers may be involved. However, their findings have not been confirmed by others. It has recently been demonstrated that *S. cerevisiae* strains carrying the single nuclear mutation *pep*4.3 or cytoplasmic petite mutations were more sensitive to the growth inhibitory effects of ethanol (1061, 1062). Although the basis for this increase in ethanol sensitivity in these mutants is not known, it is thought to result from a generalized change in the yeast membrane (1059).

Ethanol tolerant strains are often smaller in size than less tolerant strains (1013). Reduction in size increases the surface area/volume ratio, which effectively allows, for greater excretion of ethanol from the cell. As long as the rate of ethanol production does not exceed the rate of excretion, fermentation proceeds unhampered. Ethanol excretion is simply by diffusion and follows a concentration gradient (767). Therefore, as ethanol is excreted into the medium, this buildup of extracellular ethanol will cause a decrease in the rate of alcohol departure from the cell. However, it has also been inferred that the loss in yeast viability late in the fermentation correlates well with the reentry of previously excreted ethanol (767). It thus appears that for fermentation to take place a balance between three factors is required (767): 1) physical effect of ethanol on membrane fluidity (tightening), 2) fluidity of membrane due to unsaturated fatty acids (loosening), and 3) ethanol excretion and reentry. Since high temperature itself, causes additional physical and biological changes in the membrane fluidity, it is no surprise that the combined effects of high temperature and ethanol are complex. This will be considered in the next section.

c. Yeasts at High Temperature

Ethanol toxicity represents an important biological limit to the fermentation industry. This problem is further accentuated at higher temperatures. Navarro and Durand (1015) studied the effects of temperature on ethanol accumulation in *S. uvarum (carlsbergensis)*. Growth was arrested when a critical intracellular ethanol concentration had been reached and this intracellular accumulation was greater at higher temperatures. Navarro (1016) also concluded that the high intracellular ethanol concentrations were a consequence of resistance to diffusion through the membrane to the outside. Hence, at elevated temperatures, the rate of ethanol production increased faster than the rate of excretion.

A number of other studies indicate that yeasts are more ethanol intolerant at higher temperatures (302, 558, 560, 1003, 1014, 1015, 1027). However, recently Brown and Oliver (966, 1059) reported that a *S. uvarum (carlsbergensis)* strain became more resistant to inhibition by ethanol at higher temperatures. Possibly, this was due to the greater inhibitory effect of ethanol on growth at high temperature, such that more carbon was available for fermentation (966). Brown and Oliver (1059) reported that the effects of ethanol on yeast were complex as seen by the different inhibition constants for growth, cell viability, and fermentation rate. Their results demonstrated that the inhibition kinetics for growth were more complex than fermentation. This was due to a combined effect of ethanol on reducing both the rate of growth and cell viability. It was also shown that cell viability is the most important constraint on ethanol tolerance (964). Furthermore, short term adaptation to ethanol retarded the loss in cell viability on exposure to ethanol (1063). However, it has been observed that ethanol may not necessarily kill the cells but rather temporarily inactivates them in a reversible manner (1017).

Brown and Oliver's (964, 966, 1059) results also have important implications in the selection of ethanol tolerant mutants. Most selection procedures for ethanol tolerant yeasts involve the exposure of cells to progressively higher ethanol concentrations. Using this procedure, Ismail and Ali (1064) found no significant increase in ethanol tolerance. Although cell viability may be enhanced in the presence of ethanol, it does not necessarily result in isolates that can grow or ferment better in the presence of high ethanol levels. This may partially explain the poor fermentation ability of ethanol tolerant strains of thermophilic ethanologens.

Gray (558) reported that CO_2 production increased with alcohol tolerance. Watson (1065) examined a continuous culture of *S. cerevisiae* at maximum growth rate by controlling the level of CO_2 production. Brown and Oliver

(1059, 1066, 1067) recently devised a continuous selection feedback control circuit, whereby ethanol was added to a culture of *S. uvarum (carlsbergensis)*, when the fermentation rate of cells increased. Fermentation activity of the continuous culture system was based on the rate of CO_2 production, which was monitored using an infared analyzer. This signal was fed to a potentiometric controller which regulated a pump that added ethanol to the fermentor once the culture produced more CO_2 (indicative of enhanced fermentation and ethanol tolerance). After about one month the amount of ethanol added to the culture more than doubled from 2 to 4.7% (w/v) (1066). Although the cells were only exposed to 4.7% (w/v) ethanol, they fermented at twice the rate of the parent in the presence of 10% (w/v) ethanol. This feedback system should be generally applicable for the selection of isolates with improved tolerance to other inhibitory physical or chemical environments. The traditional method of raising the temperature after growth may only select for yeasts which have enhanced viability or are capable of growth at higher temperatures. However, the operation of this continuous feedback selection system at higher temperatures may prove useful for the selection of mutants capable of fermentation at elevated temperatures.

Over the last decade van Uden and co-workers have explored the targets and mechanisms of action of ethanol on the temperature relations of yeast. This work has been extensively reviewed (1031, 1068, 1069). Previous results suggested a correlation between the temperature functions of thermal death and the maximal temperatures for growth (564). Addition of varying concentrations of ethanol to strains of *S. cerevisiae* (mostly petites) or to *K. fragilis* shifted the temperature profile of growth and death to a lower range without disrupting it (1070-1073). The resultant decrease in the T_{max} and T_{opt} for growth, and increase in the T_{min}, depended upon the ethanol concentration. For a given amount of ethanol, each yeast was characterized by a temperature profile of maximum ethanol tolerance for growth (1071). As long as ethanol did not lower the T_{max} below the process temperature, the specific growth rate was greater than the specific death rate. However, at temperatures above and below the temperature plateau of ethanol tolerance, the yeast cultures became increasingly more sensitive to an ethanol-enhanced thermal death (1070, 1071). This phenomenon offers a reasonable explanation for sensitivity of growth to ethanol at high and extremely low temperature fermentations (1031, 1069).

Leao and van Uden (1046) suggested that alkanols enhanced thermal death by acting in a non-specific manner - such that the same amount of heat led to a greater amount of disorder in the activated target molecules. The target sites were made more heat sensitive with higher ethanol concentrations. There was also a correlation found between the lipid buffer partition coefficient of alkanols and its capacity for death enhancement (1046). This suggested interactions with hydrophobic regions, and that, thermal death sites were associated with a membrane system (1046). Evidence has accumulated which suggests that the sites which determine the T_{max} are identical with or have a common thermosensitive site with their thermal death site (1074, 1075).

Sherman (829, 830) first suggested that the thermal death sites of *S. cerevisiae* were located in the mitochondria since ethanol induced respiratory-deficient or petite mutants. Evidence discussed earlier, (Section IX, 1d) suggests that sterols may play a role in the development and maintenance of respiratory competencey in yeasts. More recent results show that both ethanol and high temperature enhance petite mutations. These observations suggest the inner mitochondrial membrane as the target site for ethanol enhancement of thermal death (833, 834, 1074, 1076). This may explain the greater requirement for ergosterol at elevated temperature (895, 896). The target site for the ethanol-enhanced increase of the T_{min} for growth is still unknown (1031).

It is also conceivable that fermentation at high temperature results in cell suicide for other reasons. At high temperatures, the requirement of saturated fatty acids for a rigid membrane utilizes NADH reducing power (1077). Thus, the cell may avoid producing ethanol at high temperature, since it may not have sufficient reducing power to reduce acetaldehyde, a toxic substance to the cell.

As discussed earlier (Section IX, 2a), cells can respond to the physical effects of ethanol (tightening of membrane) by fluidizing the membrane or by metabolizing this alcohol (402). It is possible that at intolerable ethanol concentrations the cells cannot fluidize the membrane further and that the rigid membrane contributes to cell death. This can be due to the toxic effects of trapped intracellular ethanol and/or to less active membrane-bound enzymes. Cells can also respond to the physical effects of high temperatures (loosening of the membrane) (1078), by changing their fatty acid composition to produce a more rigid membrane. Unlike the biological response to ethanol at higher temperatures, it is possible that cells have no means of shutting off this biological response. The result is a very rigid membrane and cell death for the same reasons mentioned above. In this regard, the cells would have no means of fluidizing their membranes in response to ethanol at high temperatures, as they do at lower ones. Thus, the synergistic physical effect of ethanol on membrane rigidity might explain the greater sensitivity of cells to ethanol at high temperatures. The hypothetical model illustrated in Table 38 could conceivably explain comparable detrimental effects of very high temperatures in the absence of ethanol, to low ethanol concentrations at high temperature, to high ethanol concentrations at low temperatures. It is presently not known whether yeasts have any means of lowering their internal temperature. Future studies are required to determine if there is a temperature gradient across the cell similar to that found with pH (1079, 1080).

3. Genetic Improvement

a. Yeasts

Although thermotolerance has been recognized as an important trait (340, 1081), there have been few reports of selection for thermotolerant yeasts. There have been some successful attempts to adapt yeasts to higher temperature. By cultivating *S. cerevisiae* at progressively higher temperatures, Loginova *et al.* (820) obtained yeasts which fermented most carbohydrates at 40°C. Another approach for rapid selection of thermotolerant yeasts might be to look for survivors from a gentle heating process. It would be expected that an increase in resistance to thermal inactivation be accompanied by an increase in growth temperature (424). El-Sheikh Idris and Berry (575) showed that thermotolerant yeasts were resistant to a 15 minute incubation at 55°C as compared to susceptible controls. Kvasnikov *et al.* (576) found that prolonged continuous cultivation in a chemostat at high temperature with a gradual increase in dilution rate was an effective procedure in selection for thermotolerant strains. However, Minami *et al.* (571) found that continuous culture often resulted in the loss of the desired yeast and selection was found to be more effective in test tubes in a temperature gradient incubator. Demchenko and Kobrina (166) examined mutagenesis as a means to generate thermotolerant yeasts. UV irradiation was found to be more effective than ethyl methane sulfonate. With UV treatment, 6 stable thermotolerant mutants capable of normal growth at 43.5°C were isolated.

As far as the author is aware, there have been few published reports on the selection of strains that produce and tolerate high concentrations of ethanol at elevated temperatures (Section VI). The Molasses Fermentation Division of the By-Products Dept., Philsugin of the Phillipines, developed an extensive screening and acclimatization program for thermotolerant high ethanol-producing yeast strains (592). They subjected a number of *S. cerevisiae* strains to a series of invigorations in pineapple juice, a medium rich in growth-stimulating substances (592). The promising isolates were then grown in this medium at 32-33°C for 60 days with periodic transfers. Thereafter, the temperature of the medium was gradually increased 1°C after every 60 days, and fermentation efficiencies measured in molasses medium. Their best 3 acclimatized isolates (Y-17, Y-24 and Y-26) were then tested under plant scale conditions at ~40°C in 18 successive repeated fermentation runs. It was noted that the fermentation efficiency of each strain at high temperatures improved somewhat with adaptation (Table 18). For example, at 40-41°C, the fermentation efficiency in strain Y-26 increased from 75% to 83.4%.

Since ethanol intolerance is accentuated at high temperatures any method to improve yeast alcohol tolerance could theoretically increase the ability to ferment at high temperatures. This was in fact the case in a number of studies (Sections VI, 2).

As noted earlier (Section IX, 2c), Brown and Oliver's (1066) novel continuous feedback selection system may be useful for the selection of thermotolerant mutants capable of fermentation at elevated temperatures. Alternative strategies for enhancing thermotolerance and/or ethanol tolerance in yeasts include the following: spheroplast fusion (Section VI), cloning of hsp's (Section IX, 1e), and cloning of regulatory genes for lipid synthesis (Section IX, 2b).

b. Bacteria

If products of industrial interest cannot be economically produced with a thermophile, the transfer and expression of a desired gene product from them into a mesophile, may be an alternative solution to this problem. In 1980, Nagahari *et al.* (148) showed that genes from thermophiles could be cloned and expressed in mesophiles using standard methods derived from work with mesophiles. This offers the possibility of increased productivity of thermostable enzymes by virtue of the gene dosage effect (83). Various genes of interest from thermophiles (eg., penicillinase, α-amylase, protease) have recently been cloned and/or expressed in *E. coli* or *B. subtilis*, bacteria which are genetically well defined (1082-1087). Often the full thermostability of these gene products is expressed (1088, 1089); however, sometimes it is restricted and/or lost with time in the transformed mesophile (148, 1083, 1090). Recent interest in cellulose degradation has resulted in cloning of the cellulase genes of *C. thermocellum* into *E. coli* (Section VII, 1e).

Recovery of thermophilic products from transformed mesophiles is facilitated through simple enrichment with thermal separation. *E coli* cells harboring a recombinant plasmid from *T. thermophilus* for expression of 3-isopropylmalate dehydrogenase activity, produced seven times more of this thermostable enzyme than the parent (149). In addition, a four-fold purification of this enzyme was made possible, by simple heat treatment of the crude extract.

In regards to growth at elevated temperatures, successful transformation of *B. subtilis* (T_{opt} 37°C) to growth at high temperatures (55-70°C) using DNA from thermophilic *Bacillus spp.* has been achieved (42, 1091-1094). This is consistent with a study in 1961, which reported that *B. stearothermophilus* produced a thermophilic factor that enabled a mesophilic *Bacillus sp.* to grow at elevated temperatures (1095). This factor was found to be dialyzable, heat stable, and destroyed by deoxyribonuclease, ribonuclease, pepsin, and papain. Although nucleic acid preparations from thermophilic bacteria were not effective, it was suggested that the factor was a nucleoprotein. A substance having similar properties may be present in the autolysates prepared from *S. cerevisiae*, for growth in a medium containing this autolysate was necessary to maintain thermophily with repeated transfers (1095). In contrast, the thermophilic principle from *B. stearothermophilus* was not required to maintain thermophily with each transfer, suggesting the occurrence of transformation. A possible explanation for these intriguing findings might be that the thermophilic factor, and possibly even the yeast hydrolysate, still contained some viable spores of *B. stearothermophilus*. It is also noteworthy that others found greater requirements for yeast extract for growth at high temperatures (Section V).

It has been hypothesized that transformation involves a small number of genes controlling a wide range of pleiomorphic effects (42, 490, 1092). Possibly changes in the protein synthesis ability of thermophilic bacteria at the ribosomal or tRNA level, and hence at the translational level, result in thermostable enzymes (1092). Ch'ih (1096) has recently initiated a study to determine the molecular mechanisms of thermotolerance in a transformed *B. subtilis* strain; the number of genes responsible for heat stability are to be determined and the genes mapped. Welker (1097) has reviewed the physiological factors affecting transfection and transformation in *B. stearothermophilus*.

Many antibiotic resistant plasmids have been isolated and characterized from thermophilic *Bacillus* (1098-1104) and *Thermus spp.* (1105-1108). In contrast to aerobic spore-formers, only a few plasmids have been reported in thermophilic anaerobic bacteria. A plasmid was found in the homoacetate-producing bacterium *C. thermoaceticum* (1109) and cryptic plasmids were recently discovered in unidentified thermophilic anaerobic bacteria (544, 1110). The presence of phages has also been reported for several thermophiles (8, 1097).

In comparison to mesophiles, few genetic studies have been conducted with thermophiles. Various mutants of thermophiles (eg., ethanol tolerant, LDH negative, and starch resistant) discussed throughout this review have been produced by standard procedures of adaptation, mutation, and selection. It would clearly be advantageous if foreign genes could be transferred into thermophilic bacteria. The thermophile might then produce new desirable products and at the same time offer many of the advantages of thermophilic microorganisms (Table 4). In addition, the employment of thermophilic bacteria as hosts for genetic transfers would pose less of a biohazard, since at room temperature thermophilic organisms are generally inactive (83). A vector with suitable cloning sites that could replicate in the thermophile and express a selectable marker such as antibiotic resistance would be required (369). However, since DNA from mesophilic organisms and selectable markers expressing resistance can denature at high temperatures, this may pose a problem. Nevertheless, there has been some success in vector development for thermophiles. Imanaka and co-workers (83, 779) have reviewed applications of host vector systems in thermophilic *Bacillus spp.* and they have found plasmids that can serve as a shuttle vector between *B. stearothermophilus* and *B. subtilis* (1085, 1111).

It was previously discussed (Section IX, 1b, iii) that low ethanol yields in *T. ethanolicus* were attributed to a regulatory problem - increasing amounts of ethanol caused feedback inhibition of ADH activity. It was also reported that an ethanol tolerant mutant of *T. ethanolicus* had higher levels of ADH activity. This ADH has recently been purified and characterized, and Ljungdahl and Wiegel's research group intend to clone the gene for this ADH and ligate it to the *C. thermoceticum* plasmid (1109). It is hoped that the transfer of this plasmid into *T. ethanolicus* will increase the copy number of ADH activity and increase ethanol production, providing the ADH-catalysed reaction is the rate limiting step (Carreira - personal communication).

There may be limits to genetic and physiological factors in improving fermentation at high temperatures. Economical processes for ethanol production at high temperatures can only be effected by continued research and development of suitable process/fermentation designs as discussed in the next section.

4. Fermentation/Process Design

Batch fermentations have been operated successfully for centuries. There have been a number of novel concepts and advances in fermentor or bioreactor design emerging over the last decade. Many of these processes offer great potential of reducing capital costs for fermentation ethanol. However, most of these are still at the laboratory scale as there are few processes that have been commercialized.

There are essentially three distinct fermentor arrangements that are being used in, or being proposed for alcohol plants: batch, cascade, and continuous (346). Most conventional fermentation is by simple batch methods. The only real advancement in batch fermentation during this century has been the use of yeast recycling techniques to reduce yeast growth and thus improve fermentation (392). A cascade operation involves a number of tanks connected in series with continuous flow of media and culture from one tank to the next. It is claimed that this increases productivity because all stages except the final one operate at reduced ethanol concentrations (346, 392). Since ethanol intolerance is accentuated at high temperature, cascade processes may be suitable for thermophilic fermentation.

Continuous fermentors are individual vessels operated continuously with a constant supply of nutrients and discharge. Most often these fermentors are operated in a cascade sequence. Continuous fermentor systems may vary from the simple continuous stirred tank reactor (CSTR) (1112) to the complex ATPAL (1113) or biostil systems (1114). Computer-controlled fermentations (1115) and genetically engineered organisms pose even further engineering challenges (1116-1118).

Most studies with thermophiles have been carried out in batch fermentations. The influence of microbial physiology on bioreactor design has been reviewed (1119, 1120). Recently Sonnleitner *et al.* (13, 82, 85, 1121) examined the behavior of a number of aerobic thermophilic *Thermus spp.* in chemostats. Technological disadvantages of these strains in chemostats included: high oxygen uptake rate, instability, growth inhibition by substrates, and requirements for unknown growth factors. It would therefore be very important to know how the thermophilic anaerobic bacteria behave in chemostats, especially on an industrial scale. Landuyt *et al.* (484, 485) increased ethanol production of *C. thermosaccharolyticum* very significantly by means of special continuous dilution culture. Zertuche and Zall (696, 711) reported higher yields of ethanol from cellulose from a *C. thermocellum* continuous culture system. However, Sonnleitner *et al.* (460) found even poorer performance of *T. brockii* in continuous culture over batch culture fermentation. A mathematical model for continuous cultivation of thermophilic microorganisms was recently proposed (1122). Gilbert and Stuart (771) recently devised a small scale chemostat for the growth of thermophilic microorganisms which serves to minimize evaporation problems at high temperatures.

In the last decade, various novel bioreactors suitable for fermentation have been developed (eg, deep jet fermentor (1123), rotor fermentor (1124), etc.). To the author's knowledge none of these novel bioreactors (reviewed in 361, 1118, 1125, 1126) have been used for thermophilic fermentations. In these reviews various criteria for comparing processes are presented. It is desirable to have a mechanically simple operation, low energy input, low capital costs, near complete sugar utilization, and high productivity. Maiorella *et al.* (1125) assessed 20 different processes as to their potential for industrial alcohol production. More recently, these same workers compared 11 alternative fermentation schemes for ethanol production using molasses and cellulose as feed (372).

Coughlin *et al.* (146) have recently written a review entitled "Immobilization Technology in Alcohol Fuels Production". Some researchers have already investigated the effects of temperature on immobilized cells (1127-1129). Estival and Burstein (1130) recently used an immobilized thermophilic bacterium (strain PS3) as a source of respiratory chain for the recycling of NAD^+. They reported that recycling of NAD^+ is at least 10 times better with the thermophilic strain as compared to *E. coli*. In the future, immobilized cells may become more widely used with thermophilic organisms because of their many advantages (361, 470, 1131, 1132): anaerobic conditions are maintained, high cell density, minimal end product inhibition, accelerated reaction rate, high dilution rate without washout of culture, and equipment/design costs are kept to a minimum. Studies have shown that immobilized *Clostridium* spores are feasible for solvent production (1133).

The high temperatures of thermophilic fermentation facilitates ethanol recovery by distillation and/or by continuous removal of ethanol from the fermentation vessel by various novel processes. These novel systems of ethanol recovery will be reviewed in the next section.

a. Systems of Ethanol Recovery

Strain development probably has its limits to solving the ethanol tolerance problem. Another means of alleviating this problem is by continuously removing ethanol from the fermentation broth by on-line extraction procedures using adsorption columns, membranes, solvent extraction, vacuum distillation, and gas stripping. Many of these procedures have been developed simply as an alternative to distillation, with the hope of lowering energy costs. Most of these techniques for selective ethanol removal have been reviewed elsewhere (368, 387, 620, 1120, 1125, 1134), and therefore they will only be briefly discussed here.

Distillation is the current method of removing ethanol from the fermentation broth, (reviewed in 1135, 1136). It is an energy intensive procedure (dependent on the amount of alcohol in the liquor), and it frequently uses more energy than is available from the ethanol recovered. Distillation costs become more favorable when fermentation broths contain >5% ethanol (w/v), especially in the 7-9% range (1137). Furthermore, an increase in fermentation temperature would lower distillation costs further.

i. Solvent Extraction

Over the last few years, a considerable effort has been devoted to developing various solvent extraction systems. Solvent extraction procedures offer the possibility of reducing energy requirements for ethanol production through the exploitation of its physical properties. Liquid-liquid extraction is particularly attractive for large scale operations because of the ease with which it can be scaled up, and its tolerance for feed stream impurities such as undissolved solids (1138).

To be successful in ethanol removal, an extractant should have the following properties (382, 743, 1125): 1) selective for ethanol so that its concentration will be higher in the extractant phase i.e., high distribution coefficient for ethanol, 2) non-volatile relative to ethanol, so that the energy expenditure for product recovery from extractant is minimized, 3) immiscible in media to allow for easy phase separation and to minimize extractant losses, 4) non-toxic, both to microorganisms and animals, 5) relatively inexpensive. Unfortunately no extractant having all the required properties has thus far been found.

It becomes apparent that solvent systems which exhibit higher ethanol distribution coefficients (i.e., polar solvents) are also more soluble in water (1120, 1138). More important however, is the fact that these polar solvents (eg., dodecanol, chlorobenzene) are highly toxic to the cell (382, 1120, 1139). Nontoxic solvents on the other hand (usually non-polar and >12 carbons), have a low distribution coefficient for ethanol (1139). Therefore, in order to better separate ethanol, recovery processes require high solvent circulation rates and more efficient configurations such as countercurrent flow systems with multiple stages (705, 1138).

Tedder and his colleagues (1138, 1140, 1141) recently developed an energy efficient low cost liquid/liquid solvent extraction system. The fermentation broth is treated with a solvent in a countercurrent cascade using a solvent with a high boiling point, that selectively removes the ethanol from the aqueous phase. Tedder and co-workers are currently investigating the possibility of using a blended solvent (Isopar-M and Tridecyl alcohol) for ethanol recovery (Tedder - personal communication). Since the optimal temperature for this blended solvent is 70°C, they are planning to use this solvent system in conjunction with a mixed culture of thermophilic bacteria (with similar T_{opt}) for alcohol and acetate production from municipal wastes. Preliminary results show that this solvent had no effect on cell morphology or activity of *C. thermocellum* and *T. ethanolicus*. However, in some cases, the cultures which are produced at higher pH's appeared to form stable emulsions. To a certain extent, this can be controlled by substrate concentration; however, additional research is clearly needed in this area (Tedder - personal communication).

Weigel (1142) has suggested that the recovery of ethanol from the fermentation broth through solvent extraction does not seem feasible for thermophilic processes using wastes. Since interfering materials, possibly deleterious, might also be extracted from the wastes, Wiegel believes that methane production from waste is a more practical alternative (1142).

It may be possible to couple liquid/liquid extraction with extractive distillation (1138). Aqueous two phase extractive bioconversion processes continually partition ethanol from the bottom phase to the top phase (1143, 1144). This prevents ethanol buildup in the bottom phase, where the bioconversion takes place.

ii. Vacuferm

Inhibition due to volatile products like ethanol can be potentially overcome by conducting the fermentation under reduced pressure and distilling off the alcohol as it is produced. The vacuferm process was developed concurrently in two laboratories (1145, 1146). When combined with continuous fermentation and recycle of a yeast cream to maintain high cell densities, very rapid fermentations were possible.

Ramalingam and Finn (1146) demonstrated that it was possible to ferment a three fold higher molasses concentration in only one third the amount of time needed in a conventional process (ethanol productivity of 12.5 g/ℓ/h). Independently, Cysewski and Wilke (1145) developed a similar vacuum system but in more detail. Using a 33.4% continuous glucose feed and a total pressure of 50 mm Hg (~.06 atm), ethanol productivities achieved were as high as 82 g/ℓ/h and 40 g/ℓ/h with and without cell cycle, respectively. Conventional processes only allowed 29 g/ℓ/h (1145). Some feel that the major savings come from continuous operation and cell recycle rather than the use of vacuum (1120).

Besides lowering distillation costs and increasing productivity rates, no cooling is required for this process i.e., the heat of fermentation is used directly for distilling off the alcohol (1145). In fact, the heat of fermentation may be insufficient to sustain isothermal evaporation in a reduced pressure system (387). In this regard, the use of thermotolerant yeasts would improve fermentation under reduced pressure or would permit higher pressure in the fermentor. Both these processes would further reduce energy costs. As discussed earlier, removal of H_2 from a *C. thermocellum* culture by agitation, resulted in lower ethanol yields (685). Since a vacuferm process or its variatons (Azeoferm, Flashferm) (518, 1113) will also remove H_2 from the culture, it appears that alcohol production with *C. thermocellum* in mono- or co-culture may not be carried out efficiently by fermentation under vacuum. Preliminary results showed this to be the case (14). It is possible to incorporate a recycling of the hydrogen gas generated during the fermentation as was proposed for the Penn/GE Process (745), or for Mulholland's CO_2 stripping process, discussed in the next section. In a patent (734), Zeikus claimed use of low amounts of hydrogen added in the head space, during co-culture fermentations of cellulosic biomass. Grethlein and co-workers at Dartmouth College, Hanover, New Hampshire, are about to undertake a study to examine the performance of *C. thermocellum* in a small scale vacuferm (Grethlein - personal communication).

A number of physiological as well as engineering problems arise in a vacuum process. In view of the highly anaerobic conditions and elevated growth temperatures, special nutrients are needed (1120). It is also necessary to sparge the vacuferm with pure oxygen. Since, the vacuum operation works under reduced pressure there is an increased likelihood of contamination (1125). In order to maintain viable cultures it is also necessary to continuously bleed the fermentation broth to remove non-volatile compounds. The major drawback of the vacuferm is the great energy consumption involved in pumping the vast volume of CO_2 from the vacuum (1120, 1145). However, schemes have been proposed for accomplishing this economically (371). As far as the author is aware, however, no industrial plant has been built according to the vacuferm principle.

iii. Gas Stripping Ethanol Purifier

Lindsay Mulholland (1147) of the Ontario Research Foundation, Toronto, Ontario, examined the potential of removing ethanol from a fermentation broth by stripping it with various gases such as CO_2. Critical Fluid Systems developed a process (Supercritical Fluid Extraction) that uses near supercritical CO_2 (total pressure of 1000 psi) to recover ethanol from dilute solutions in a counter current liquid/liquid extraction system (1148). Hartley (369, 533) and his colleagues proposed a scheme using recycled CO_2 for ethanol stripping with their *B. stearothermophilus* process (Section V, 3). Carreira and Ljungdahl (441) found that a recycled stream of oxygen-free nitrogen gas removed the ethanol from fermentations of glucose by *T. ethanolicus* at 70°C and this is covered in a patent (467).

Mulholland (1147) recently developed a gas stripping ethanol purifier converted to a continuous fermentor system for the production of ethanol by a yeast. Since this system has many potential advantages over other systems, and is most suitable for thermotolerant or thermophilic microorganisms, it will be described in more detail (1147, Mulholland - personal communication). Inclusion of a draft tube in an 11 *l* glass cylindrical vessel provided a cyclical liquid flow in the fermentor. A nutrient sugar solution (~40% w/v glucose) was pumped into the vessel at a continuous rate (~5% w/v sugar in steady state) to match the liquid loss due to volatilization of ethanol and water. The CO_2 produced by the yeast was pumped back into the fermentor and was used for stripping off the ethanol (~2% w/v in broth) A pressure of ~3 psi (pCO_2) was necessary to push the liquid and gas through the draft tube; excess CO_2 was vented. Ethanol was removed from the gas phase by condensers

(~10-20% w/v ethanol in condensate). Since the minimal successful temperature for stripping was 40°C, Mulholland (1147) tested two strains of *S. cerevisiae* for fermentation at this temperature. *S. cerevisiae* strain ATCC 4126 grew and fermented well in his system at 40°C; (>90% fermentation efficiency) however, in the absence of gas stripping, no growth or ethanol production was seen at this temperature.

Mulholland's fermentor system was operated successfully over a three month continuous period. Productivity rates were low ~0.6 g/ℓ/h, and some contamination and viability problems were encountered. This was attributed mainly to the lack of good automatic controls (sugar feed, liquid level, flow of gas and especially pH). The major concern in this process is the effect of CO_2 on microbial growth and fermentation. It is evident from Jones' extensive review (1149) that all microorganisms' growth and metabolic processes are affected by CO_2. At various concentrations, depending on the specific organism, both CO_2 and HCO_3^- are capable of inhibiting both the growth rate and cell yield (559, 1150, 1151). They do this in large part by altering membrane properties and enzyme activities (1149).

Suggestions have been made that the use of elevated pCO_2 may result in minimizing cell biomass and maximizing viability and product yield (1149). Norton and Krauss (1152) reported that the glycolytic flux for anaerobic ethanol production of *S. cerevisiae* was insensitive to pCO_2 values <4 atm. However, others have shown that a pCO_2 of 2 atm can decrease the fermentation rate (1151). It becomes apparent that the metabolic consequences of elevated pCO_2 upon fermentation of microorganisms are complex and not well understood. The fermentation will be affected by various factors: temperature, pH, osmotic pressure, strain, ionic strength, etc. For example, the inhibition by CO_2 is more marked in the presence of glucose or ethanol (1149). However, in Mulholland's continuous system, only low levels of these solutes are present at any one time. Furthermore, the higher temperatures may make the CO_2 less inhibitory by decreasing its solubility.

The gas stripping ethanol purifier offers many potential advantages over conventional systems (Table 39). By continuously removing ethanol, it may alleviate ethanol toxicity and result in higher productivities. However, this remains to be seen (see Section IX, 4a, v). Although equipment costs are not known, it is believed that capital costs will be lower (Mulholland - personal communication). Furthermore, energy costs will be significantly lower since distillation costs will be minimal and there would be no need for cooling or mixing.

The use of a thermotolerant or thermophilic microorganism in this system would further reduce overall costs. Increasing the fermentation run temperature would lead to a greater overall stripping efficiency. The ethanol uptake per unit volume of gas sparged into the system increases by ~2-fold per 10°C rise in operation temperature (1147). Furthermore, at higher temperature, relatively less uncondensed vapor would be recycled. Mulholland's system might work well with *C. thermocellum* in mono- and co-culture thermophilic fermentation of cellulosics. It was mentioned earlier, that processes like the vacuferm were not suitable for *C. thermocellum*, because they removed the H_2 gas, which resulted in lower ethanol yields (685). Since the ethanol purifier continuously recycles most of the gases back into the fermentor, theoretically, *C. thermocellum* should not have this problem. Although it is still in the early developmental stages, this novel fermentation design appears very promising for thermotolerant or thermophilic microorganisms. It remains to be seen how they would behave in this system in reality (i.e., long term effects of stripping process, etc.). Preliminary work has shown that Mulholland's system may have good potential for acetone butanol recovery (Mulholland - personal communication) or for that matter, any fermentation product that is sufficiently volatile to be stripped.

iv. Other Systems of On-Line Extraction

Various membrane processes of ultrafiltration or reverse osmosis may some day find application in continuous alcohol removal (620, 1153). Ideally, one is looking for a membrane that has high flux, high selectivity, material compatibility, low cost, and ease of handling and maintenance (1154). However, the commercial separation of ethanol and water by a membrane process is still not presently attractive using commercially available membranes (1154).

Ethanol can be recovered from the fermentation beer by selective adsorption techniques which usually consists of 2 steps. Ethanol is first separated by adsorption on solids (eg., activated carbon, crystalline zeolite) (1155, 1156). Subsequently, ethanol is removed from the pores of the solid material by heating or gas stripping (705, 1156, 1157). Often the sugars and dissolved solids can reduce the ethanol loading onto the adsorbent. Furthermore, there are complications due to inherent problems usually associated with recovering ethanol from the solid support. Ladisch and Dyck (1158) have reported a novel way of recovering ethanol which gave a positive energy balance - dehydration of ethanol by using cellulosic materials (starch, corn, etc.).

v. Cautionary Note

Kinetic studies have shown that progressively increasing amounts of ethanol added to a culture inhibit fermentation (Section IX, 2). Therefore, it has been assumed that if ethanol was continuously removed from the fermentation broth, the ethanol production rate would be increased. This was confirmed by the vacuferm processes discussed previously. (1145, 1146). Although, no one has reported removing ethanol continuously from a thermophilic culture, one might expect that this would lead to a further increase in productivity. This is because ethanol is much more toxic to the cell at high temperatures.

Recently, Lencki *et al.* (1159) reported that on-line ethanol extraction during yeast fermentation (in repeated experiments using several hydrophobic adsorbents), did not enhance ethanol productivity as one would have expected. After carefully analyzing Cysewski and Wilke's results (1145), it appears that their claims of higher ethanol production rates in the vacuferm process were due to increased cell numbers in comparison to those in the controls. When Lencki and co-workers (1159) standardized Cysewski and Wilke's (1145) results on the basis of cell number, there was essentially no difference in ethanol production rates. Other papers reporting higher productivities did not report cell numbers, so fair comparisons cannot be made (Robinson - personal communication). If this is a reproducible phenomenon with other microorganisms and in other systems, then it would have important implications in current research in this area. Therefore, it should be looked at more carefully. Perhaps, removal of extracellular ethanol does not increase the outward flow of intracellular ethanol, the truly toxic metabolite from the cell.

X. GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER STUDY

1. Microbial Features

a. Bacteria

General and specific features of thermophilic bacteria make them of significant potential for many industrial processes including ethanol production. To date, thermophilic bacteria have not been employed in commercial ventures, but the potential exists for them to eventually compete with yeasts. In the last few years a number of concerted research efforts in academic institutions (MIT, Wisconsin, Georgia), have led to very promising results. The advantages of thermophilic bacteria have only recently been recognized. Before realistic assessments can be made, more intensive basic and applied research will however be required. This will entail a cooperative multidisciplinary research effort from the physiologist to the engineer at the industrial, governmental and academic levels.

"Thermophily" has the potential to be superior to "mesophily" for ethanol production, and in view of reductions in energy and production costs, thermophily could also be suitable for many other biotechnological processes. The wide substrate spectrum, high metabolic rate, and reduced requirement for temperature control render thermophilic bacteria especially attractive as potential solutions to environmental problems (sewage treatment, composting, leaching processes, coal desulfurization, etc.). Furthermore, these organisms have potential as sources of thermostable industrial enzymes. Although there are currently very few enzymes from thermophiles produced commercially, they will probably replace mesophilic enzymes in the not too distant future due to various advantages offered. One particular advantage is that after their genes have been cloned into a mesophile, thermophilic enzymes can be easily purified by thermal separation. Immobilized systems would be particularly useful in exploitation of the metabolic capabilities of thermophiles. Although thermophilic bacteria are not suitable for the production of other alcohols, they have great potential for acetic acid production.

Since raw material accounts for up to 70% of the final ethanol cost, major breakthroughs in fermentation ethanol will depend upon the use of less expensive feedstocks such as cellulosic biomass. If a fermentation process for ethanol production is to be economically feasible, the direct conversion of cellulose to ethanol is essential. Currently available yeasts cannot degrade cellulose, consequently the biomass must first be hydrolyzed by acid or enzyme, an additional cost factor. However, efforts are ongoing to clone cellulase genes into yeasts and this area should continue to develop further. In addition, no yeast has been found to efficiently ferment xylose in a demonstrated economical process. Some thermophilic ethanologenic anaerobic bacteria in co-culture with the cellulolytic organism, *C. thermocellum*, offer great potential to convert both the hexose and pentose fractions of cellulosic biomass directly to ethanol. High productivity rates and ethanol concentrations have been achieved from non-cellulosics in a few parent strains, but only at low uneconomical substrate concentrations (usually $\leq 2\%$ w/v). There are essentially two major problems accounting for these effects:

1) slow rates of cellulose hydrolysis due to low cellulase activity, 2) low ethanol productivity and yield due to reduced ethanol tolerance. Although significant improvements have been made in both of these areas, it is too early to predict maximum rates and yields.

Various physiological factors can influence the cellulase yield of *C. thermocellum*. For example, studies have demonstrated that the simultaneous inclusion of a thiol-reducing agent and calcium can significantly increase cellulase activity. In fact, under these conditions, the cellulase system for *C. thermocellum* has a higher specific activity on crystalline cellulose, as compared to the fungal cellulase system. However, presently, productivity (amount of protein per biomass is still low as compared to hyperproducing mutants of *T. reesei*. The cellulase system of *C. thermocellum* has received little attention compared to that of *T. reesei*. It appears that upon further study, rates of cellulose conversion may be enhanced and that the cellulase system of *C. thermocellum* might subsequently compete effectively with fungal cellulases. There is still need for improvement in the methodology of cellulase purification and standardization for quantitating cellulase activity. Experiments should be conducted with more pertinent cellulosic substrates (eg., agricultural and wood wastes), rather than on commercially-available purified cellulosics such as solka floc. The dilemma of the sensitivity of thermophilic bacteria to inhibitors present in the lignocellulosics still needs to be resolved. Studies should continue to focus more on the cellulose/cellulase complex (cellulosome). For example, purification of the "yellow substance" produced during cellulose degradation may lead to a better understanding of the importance of the cellulosome.

The further investigation of culture parameters and the regulatory mechanisms of cellulase production is recommended. Exploitation of continuous culture with cell recycling may further increase overall productivity. Strain improvement has been impeded because there is little known about the genetics of this bacterium. There have been too few attempts to improve cellulase titres by mutation and selection programs. Inclusion of non-metabolizable cellobiose analogs and other antimetabolites in the medium, may aid in the selection of hyperproducing cellulase mutants. Developments in recombinant DNA technology pertaining to these organisms are still in the early stages; however, a number of researchers have cloned cellulase genes into *E. coli*. Since the cellulase system is complex and requires several enzymes, in all likelihood, it may be necessary to clone several genes in order to obtain good cellulase activity.

Metabolic studies of thermophilic bacteria suggest that a variety of physiological and biochemical factors control the production rate and concentration of ethanol. These include parameters that influence the carbon and electron flow to other products (acetate, lactate), energy metabolism efficiency, regulation of end products, and species tolerance to each product. Ethanol yield appears to be related to the activity and regulatory properties of ferredoxin-NAD(P) oxidoreductases, hydrogenases, and FDP-activated LDH's. This can account for reduced ethanol yields and productivities under physiological conditions that raise the intracellular FDP level, and in the presence of exogenous electron acceptors.

The low ethanol yield of *C. thermohydrosulfuricum* and *T. ethanolicus* appear to be related to the ethanol-dependent inhibition of their ADH's. It was observed that ethanol tolerant mutants have higher levels of ADH activity. An important target for future research would be to study the regulatory properties of the ADH's further and to isolate improved ADH-deregulatory mutants from these organisms. A long term approach to increasing ADH activity might be through recombinant DNA technology; cloning of genes expressing ADH activity onto a plasmid from a thermophilic microorganism and the transfer of this vector into the desired thermophile might increase the ADH gene copy number and possibly stimulate further ethanol production.

Despite some interesting findings, intra- and inter- specific metabolic control in thermophilic bacteria is complex and remains poorly understood. Further improvements in ethanol yield will require research aimed at better understanding the metabolic factors that control the rates and yields of product formation. This should then provide useful insight for optimization of culture conditions, process development and strain selection. In particular, studies should focus on identifying the nutritional requirements of thermophilic bacteria. Some assessment should be made to determine whether or not the nutritional factors present in yeast extract or heart infusion broth can be fulfilled by stillage, corn steep liquor, etc.

Genetics will play an important role in the development of thermophiles for industrial use. In comparison to mesophiles, very few genetic studies have been conducted with thermophiles. Most efforts in this area have concentrated on improvement of ethanol tolerance by adaptation, mutation and selection procedures. These have resulted in modest improvements in ethanol tolerance.

Further improvements by these means will require a greater understanding of the physiological basis for ethanol tolerance, as has been initiated with *C. thermocellum*. Transfer of desired thermophilic genes to mesophilic bacteria with better defined genetic systems should provide valuable information. This will entail the isolation and characterization of plasmids, especially those from the thermophilic anaerobic bacteria. Genetic construction of thermophilic genes in mesophiles should facilitate product recovery by thermal separation. It must also be elucidated whether thermophilic bacteria are truly subject to higher mutation frequencies, and if so, how to control stability. In particular, their stability in continuous culture must be examined.

Several thermophilic bacteria have been isolated during the last few years and their attributes are just now being realized. It is difficult to rank the relative industrial potential of various thermophilic bacteria since a large body of relevant information is still required. Extensive efforts are still underway with *T. ethanolicus*, *C. thermohydrosulfuricum* and *C. thermosaccharolyticum*, the thermophilic anaerobic ethanologens which perform well in co-culture with *C. thermocellum*. With low glucose concentrations, *T. ethanolicus* produces near theoretical yields of ethanol (1.95 moles ethanol/mole glucose). It is also the easiest thermophilic bacterium to adapt to ethanol (10% v/v) and it appears to exhibit tolerance to the inhibitors in lignocellulosics. The metabolism of *T. ethanolicus* has been studied and this has led to the selection of mutants that tolerate 10% (v/v) ethanol and produce up to 3-4% (w/v) ethanol from starch. *T. ethanolicus* has a very wide substrate spectrum, exhibits an equivalent growth rate at a broad pH and temperature range, and can use pentoses and hexoses concommitantly without any indication of diauxic growth. A more intensive mutation program and an investigation of mutants selected on carbohydrate mixtures rather than starch alone, might result in isolates displaying superior performance. Subsequent research efforts should be directed toward better understanding the metabolism and genetic system of *T. ethanolicus*, and its use in novel process/ fermentation designs.

C. thermohydrosulfuricum also appears to have potential use, as it has been reported to produce near theoretical yields of ethanol from low substrate concentrations, and it has a faster doubling time than *T. ethanolicus*. Its unique membrane structure enables this bacterium to tolerate high methanol concentrations. However, it is more sensitive and more difficult to adapt to ethanol than *T. ethanolicus*, and it requires a pH drop for ethanol formation. Although it was concluded from a two year study that this bacterium, was not economically feasible for the conversion of xylose to ethanol, it has performed well in co-culture with *C. thermocellum*. Mutants have recently been isolated which are capable of producing up to 3-4% (w/v) ethanol under certain conditions. Continued studies on its metabolic regulation and genetic systems may lead to further improved strains of practical value.

Significant improvements have also been made with *C. thermosaccharolyticum*. Mutants tolerating ~5% (w/v) ethanol and capable of producing up to 4% (w/v) ethanol from xylose have been isolated. Although it performs well in co-culture with *C. thermocellum*, at present, the yields of ethanol from even low substrate concentrations are too low. This organism only produces ethanol under certain conditions (resting, nondividing or elongated cells). In comparison to the other ethanologens, relatively little is known about its metabolic regulation. Continued studies of this bacterium, especially under a condition of "special continuous dilution culture which elongates cells", might improve its potential.

Although significant understanding of *T. brockii's* metabolic pathways show interesting results, it does not appear that this bacterium will prove useful in the future for ethanol production. However, additional studies with this thermophile might lead to a better understanding of the factors involved in the metabolism of thermophilic bacteria.

A multidisciplinary approach has resulted in the conversion of the well known food contaminant, *B. stearothermophilus*, into a potentially suitable organism for ethanol production. Efforts should continue to further explore its normal control mechanisms which cause the shift to the desirable PDH pathway, and result in near theoretical yields of ethanol. Since this facultative anaerobe is presently limited by the substrates it can utilize, research into recombinant DNA approaches to widen its substrate utilization spectrum would prove useful. Genes for synthesizing several cellulose-degrading enzymes, have already been cloned into other bacteria and host vector systems have been developed for *B. stearothermophilus*. Future directions in research might be to introduce foreign genes coding for cellulolytic enzyme activity into *B. stearothermophilus*.

Other recently discovered thermophilic bacteria such as *T. acetoethylicus* or *Clostridium sp.* strain Tx should be further characterized for their fermentation or cellulolytic abilities. It is conceivable that thermophilic bacteria with more desirable properties still exist in nature, perhaps in unexplored environments. Hence, an ongoing continuous search for new isolates and the characterization and examination of their ethanol producing capabilities, is warranted.

Another organism highly ranked for ethanol production is the mesophilic bacterium *Z. mobilis*. It demonstrates higher ethanol productivity rates than yeast and understanding of the physiology and genetics of this bacterium has increased significantly in recent years. Nevertheless, it is generally agreed that it cannot compete successfully with yeast, because of the need to maintain cultures at pH ≥5, which imposes a strict requirement for sterilization to prevent contamination. Research efforts should continue to increase its ethanol and temperature tolerance and to broaden its narrow substrate utilization spectrum by genetic engineering.

b. Yeasts

It is evident that thermotolerant yeasts would offer many advantages to the fermentation industry. Energy costs (cooling, distilling, and mixing) would be minimized and theoretically, productivity and growth rates should increase. Thermotolerant yeasts would be especially attractive in tropical countries where cooling costs are very expensive, and they are paramount for efficient SSF processes currently being investigated. Thermotolerant yeasts may also be advantageous for other industrial applications such as in the wine, brewing, and baking industry. In view of their many advantages, it is surprising that so few attempts have been made to select for thermotolerant yeasts and in particular yeasts that can produce ethanol efficiently at high temperatures.

More research is needed to screen and select for yeasts capable of fermentation at high temperatures. Studies should focus on the isolation and examination of yeasts from thermal ecological niches as has been conducted with bacteria. At the same time, a selective screening for organisms with good fermentation ability (using Durham fermentation tubes) should be made from natural sources such as cacti. A particularly good source for such isolations would be sugar-containing compost (fruits, sugar cane, etc.). Future selections for thermotolerant isolates should take advantage of temperature gradient incubators. Selection of ethanol-producing strains in a chemostat at high temperature might also lead to thermotolerant isolates with good fermentation ability. Another approach would be to quantitate the number of survivors following gentle heating of the broth.

Since metabolic activity and growth rates increase with temperature, it would be expected that ethanol productivities would increase as well; however, this does not always occur since ethanol intolerance is accentuated at higher temperatures. Although theoretically, ethanol tolerant mutants would exhibit enhanced fermentation at higher temperatures, unfortunately, there have been few reported successful attempts to generate these ethanol tolerant mutants. In two cases, spheroplast fusion has resulted in fusion products that yield higher amounts of ethanol and ferment better at higher temperatures. Spheroplast fusion of thermotolerant and/or of ethanol tolerant parent strains should be conducted and the fermentation abilities of their fusion products at high temperatures examined.

To date, there has been little success in the isolation of yeasts with superior fermentation abilities by exposure of the cells to progressively higher ethanol concentrations. This may be due to the fact that ethanol affects the cell in a complex manner. To select for increased fermentation ability, ethanol should be added to the culture when the cells fermentation rate increases. It is obvious that examination of the fermentation ability on repeated transfers can be a time consuming, cumbersome task. However, a novel continuous selection feedback control system performs this in a simple manner. Ethanol is automatically added to the culture when the fermentation rate of cells increase (based on measured CO_2 levels). This system resulted in mutants that were resistant to twice the amount of ethanol that the cells were exposed to. The traditional method of increasing the temperature following growth, may only select for yeasts which have enhanced viability or are capable of growing at elevated temperatures. However, the operation of this novel continuous feedback selection system at higher temperatures might be very suitable for the selection of isolates capable of fermenting efficiently at high temperatures. It therefore deserves considerable attention.

It is apparent that to date no true thermophilic yeasts (T_{max} >50°C) have been identified. However, some species or strains are definitely more thermotolerant than others. Although insufficient studies have been conducted, it appears that many species of *Saccharomyces*, *Candida*, and *Kluyveromyces* are thermotolerant. Improvement in ethanol tolerance in the latter two genera in particular, might lead to higher ethanol productivities at elevated temperatures.

For the majority of the species examined, the optimum temperature for fermentation was shown to be ~5-10°C higher than that for growth. It has been concluded that the large differences in the growth and fermentation temperature optima would make thermophilic yeast fermentation processes impractical for industrial application. Nevertheless, it is conceivable that the differences in temperature optima between growth and fermentation may prove to be beneficial for a thermotolerant yeast fermentation adiabatic system, especially in tropical countries. A 2-step fermentation process can be visualized where initally a yeast would grow rapidly at ambient temperature (~20-30°C) and its growth rate would increase with time due to the exothermic fermentation process. The gradual rise in temperature in the fermentation medium would eventually slow down growth and enhance the fermentation rate. Although ethanol inhibits growth rates, fermentation rates, and cell viability, it has been demonstrated that the latter effect is the most important constraint on ethanol tolerance. The strategy would thus be to ensure that a large number of cells are present before the high temperature is achieved. Therefore, studies should be conducted in the future with high cell densities; in particular, immobilized cells or recycling procedures might be effective for thermotolerant or thermophilic fermentation processes.

Since many factors influence the ability of yeasts to grow or ferment at high temperature, studies should take these into consideration when comparing strains. It is known that at higher temperature, oxygen solubility decreases and nutritional requirements such as for unsaturated fatty acids increase. It is possible that a number of yeasts may be capable of growth at elevated temperatures, but have not been cultivated under optimal physiological conditions, or have not even yet been tested. Therefore, further studies examining the physiological factors affecting fermentation at high temperature should continue in a chemostat, with proper controls for oxygen, pH, etc.

Continued investigation of the effects of high temperature on the stability of cellular components (proteins, membranes and lipids, constituents in protein synthesis) is required. In particular, the temperature-dependent interaction between membrane lipids and proteins should be investigated. Efforts must also focus on the physiological basis of ethanol tolerance in yeast. Research on intracellular ethanol is necessary to determine if it is responsible for detrimental effects on the cell. It should also be determined whether ethanol acts on a specific target site such as a specific enzyme, or in a generalized fashion as an uncoupler. Studies are also required to examine the effects of ethanol on the temperature profiles of yeast. Examination of ethanol tolerance and/or productivity of ADH regulatory mutants of *S. cerevisiae* is recommended.

In order to deal effectively with the problems of high temperature and ethanol toxicity, a better understanding of the physiological principles which account for the detrimental effects of ethanol on a given yeast strain is required.

It would therefore be recommended that efforts be focused on a comprehensive physiological/biochemical/genetic study of one particular genetically-marked strain of *S. cerevisiae*. A complex study should examine the effect of various physiological factors [unsaturated and saturated fatty acids, sterols, polyols, minerals (Ca^{++}, Zn^{++}, Mg^{++}, NH_4^+), amino acids, vitamins, nitrogen sources, sugar type and concentrations, pH, ionic strength, cell density, degree of aeration and DO content, age of cells and hydrostatic pressure] on the ability of this strain to grow and/or ferment at high temperatures.

It appears that trapped intracellular ethanol due to increased membrane rigidity as a result of an increase in saturated fatty acids at high temperatures, may be partly responsible for reduced cell viability and hence, reduced ethanol tolerance at high temperature. Efforts should be devoted in particular to the regulatory control of fatty acid biosynthesis. The selection and study of fatty acid deregulatory mutants, and their characterization should provide pertinent information. Possibly, long term research efforts might aim at cloning structural or regulatory genes responsible for fatty acid synthesis, or of genes involved in synthesis of unique lipids such as those from *L. heterohiochii*. Presently, the reasons for a more rigid membrane at high temperature are not known. It is conceivable that membrane rigidity may be required for the functioning of certain membrane bound enzymes, or it may be a survival mechanism to prevent cell dehydration. However, it remains to be determined whether intracellular water loss is accentuated at high temperatures, and in addition, whether cells have the ability to regulate their internal temperature. Since the reserve carbohydrate trehalose, may function as a protective agent under conditions of stress (eg., absence of water), the effect of trehalose on thermotolerance should be studied. In a similar manner, polyhydroxy compounds (sugars, polyols, and their derivatives) which have been reported to increase thermal stability in mammalian cells, should be examined. The effects and control of glycosylation of yeast proteins on thermostability should also be carefully assessed.

Another area of potential for exploration is heat shock. Heat shock, exposure of cells to high temperature, induces a set of heat shock proteins (hsp's), which were initially thought to be responsible for the acquisition of thermotolerance in heat-stressed cells. In addition it has been reported that heat shocked yeasts are more ethanol tolerant than heat-untreated yeasts. More recent studies involving inhibition of protein synthesis with cycloheximide suggest that the hsp's may be incidental and not responsible for imparting thermotolerance. Other cellular stressors such as ethanol, metals, etc. also induce these same hsp's. Hence, studies should continue to examine whether thermotolerance can be acquired by these other factors, and if so, their proteins should be examined. Research should also continue to elucidate if heat shocked yeasts are consistently more ethanol tolerant, and if so, their abilities to ferment at high temperatures should be examined. It will be important to resolve the question of whether there is a set of hsp's associated with the acquisition of thermotolerance and/or ethanol tolerance. If so, then this offers the possibility of cloning these genes into microorganisms in order to express both desirable traits simultaneously. Fusion of the yeast heat shock gene hsp 70 to the *E. coli* β-galactosidase gene has been extremely useful in characterizing the heat shock response of *S. cerevisiae*. Future studies should continue to fuse other yeast heat shock genes and determine stimuli that induce gene expression. The effects of these stimuli on thermotolerance in yeast should then be assessed.

2. Process Features

Significant effort must also be directed towards solving the engineering problems confronting thermophilic fermentations. Since strain development may have limits in solving the ethanol tolerance problem, another solution may be to continuously remove the ethanol from the fermentation broth by on-line extraction procedures. However, in view of recently published conflicting results, it is very important to determine whether or not removing ethanol from the medium actually enhances ethanol productivity. If so, then efforts to continue in this direction should proceed.

Solvent extraction does not appear to be a feasible alternative at present since solvents which give good ethanol separation are more toxic to the cell. Likewise, other techniques such as membrane separation, aqueous phase extraction, etc., presently pose several problems. It should be deduced whether thermophilic fermentations would lower energy requirements for product recovery via reduced pressure in a vacuferm. Efforts should also continue to search for new methods of recovering fermentation products. Of the methods of on-line extraction of ethanol available, it appears that the CO_2 stripping process warrants further investigation. It exhibits potential for reducing energy costs (cooling, mixing, distilling), and unlike the vacuferm, since

H_2 gas is recycled back into the culture, it should be theoretically suitable for *C. thermocellum* in mono- or co-culture fermentations. Therefore this system, or similar systems based on this concept, should be tested with a variety of substrates and a host of thermotolerant yeasts and thermophilic bacteria. Since CO_2 can affect most microbial processes, the long term effects of CO_2 stripping on the different microorganisms remains to be determined.

Significant effort must also be directed towards solving the engineering problems facing thermophilic fermentations. Ongoing continued research and development of suitable process/fermentation designs is crucial to the development of high performance commercial bioreactors for bioconversion processes. Since most studies with thermophiles have been conducted only in tubes, flasks, or small fermentors, it remains to be seen how thermophiles will perform in controlled large scale bioreactors, including cascade and continuous culture systems. Immobilized cells and enzymes, or fixed film reactors, should also be examined, since they may improve rate, yield, and recovery of fermentation products.

Although thermophiles have many potential advantages, there are a number of aspects which need to be resolved by continued research: the effect of high temperatures on the stress of the equipment (steel fermentors, sensors, etc.), the oxygen requirements for aerobic organisms at high temperatures, effects of high substrate concentration, evaporation problems, changes in viscoelastic properties of the broth, and actual expenditures for cooling, heating and distillation. Although none of these problems appear insurmountable, they should not be disregarded. Instead engineers should be motivated to improve process/fermentation designs.

Before processes for fermentation ethanol at high temperatures are economically feasible, engineers will have to solve many new unexpected problems. For example, the importance of cooperation between physiologists, geneticists, and engineers can be demonstrated by the unexpected finding that stirring *C. thermocellum* cultures results in lower ethanol/acetate ratios. Metabolic studies have established the cause for this effect - stirring removes hydrogen, which due to the presence of specific enzymes results in less inhibition of acetate production. Improvements in processes involving *C. thermocellum* might be achieved by the selection of mutants lacking these enzymes, or with proper fermentor designs for maximizing the H_2 concentration.

The present oil glut is not a solution to the energy crisis since it is only a matter of time before our nonrenewable petroleum supply is reduced and prices begin to rise once again. The fermentation of renewable resources to ethanol can regenerate our fuel and chemical feedstocks; however, the economics of this are presently not feasible. Production of ethanol by fermentation processes at high temperatures can offer significant cost reductions, but considerable more basic and applied research is necessary. In addition to the need for joint efforts by scientists from various disciplines, a cooperative effort will be required between government, industry, and academic institutions. It is evident that the success of any process to employ microbial fermentations at higher temperature will require adequate incentives in terms of time and investment on the part of the government and commercial backing by the private sector. In essence, what is really necessary is a long term research mandate in many scientific disciplines, supported by sufficient funds and the required manpower. Such a mandate would make thermophilic bacteria and/or thermotolerant yeasts of tremendous potential in commercial fermentation processes.

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Table 1

POSSIBLE FACTORS AFFECTING GROWTH AND/OR FERMENTATION AT HIGH TEMPERATURES

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1. Species - Strain type (genetic constitution).
2. Degree of cell viability.
3. Nutrients - vitamins, cofactors, minerals, amino acids, nitrogen source, fatty acids, etc..
4. Substrate type.
5. Substrate uptake.
6. Substrate concentration and solubility (feeding rate).
7. Water activity (osmotic pressure).
8. Cell density (pitching rate).
9. Ionic strength.
10. pH.
11. Presence of inhibitory substances.
12. Product formation rate and degree of tolerance (ethanol, acetic acid, lactic acid, H_2, CO_2, etc.).
13. Degree of aeration and mixing (dissolved oxygen content).
14. Hydrostatic pressure.
15. Physiological state of cells (age, morphology, storage conditions).
16. Presence of contaminants (chemical and/or biological).
17. Redox potential balance.
18. Energy metabolism efficiency (metabolic pathways and regulatory controls).

Table 2

CLASSIFICATION SYSTEMS FOR THERMOPHILIC BACTERIA

Term	Definition	Year	Ref
Thermophiles	$T_{min} > 25°C$	1907	5
Orthothermophiles	$T_{max} > 60-70°C$		
Thermotolerants	$T_{max} \approx 50-55°C$		
Strict thermophiles	$T_{opt} > 55°C$, $T_{min} \geq 37°C$	1927	6
Facultative thermophiles	$45°C \leq T_{opt} \leq 55°C$		
Thermotolerant species	$37°C \leq T_{opt} \geq 45°C$		
Thermoduric mesophiles	$20°C \leq T_{opt} \leq 37°C$ and survive pasteurization (62.8°C for 30 min)		
True thermophiles	$T_{opt} \approx 55-60°C$	1945	7
Stenothermal thermophiles	$T_{min} > 28°C$, $T_{max} < 60°C$		
Eurithermal thermophiles	grow at 60°C and at 28-30°C		
Strict or obligate thermophiles	T_{opt} 65-70°C, $T_{min} > 40-42°C$	1969	8
Facultative thermophiles	T_{max} 50-65°C, T_{min} room temp		
Thermotolerant organisms	T_{max} 45-50°C, T_{min} room temp		
Hypothermophilic	$T_{max} \geq 65°C$	1973	9
Caldoactive bacteria	$T_{max} > 70°C$, $T_{opt} > 65°C$, $T_{min} > 40°C$	1975	10
Thermophilic bacteria	$T_{max} > 60°C$, $T_{opt} > 50°C$, $T_{min} > 30°C$		
Thermotolerant bacteria	$T_{opt} < 40°C$, $T_{max} \geq 43°C$	1980	11
Thermophilic bacteria	$T_{min} \geq 25°C$, $T_{opt} \geq 40°C$, $T_{max} \geq 50°C$		
Extremely thermophilic	$T_{min} \geq 35°C$, $T_{opt} \geq 65°C$, $T_{max} \geq 70°C$		
Facultative thermophiles	$T_{min} \leq 45°C$, $T_{opt} > 45°C$	1981	12
Obligate thermophiles	$T_{min} > 45°C$		
High thermophiles	$T_{opt} \geq 75°C$		
Extreme thermophiles	near boiling point of water		
Thermoduric	heat-resistant cells		
Eurythermal	broad temperature range		
Stenothermal	narrow temperature range		
Thermotolerant bacteria	$T_{min} < 30°C$, $T_{opt} \leq 55°C$, $T_{max} \leq 65°C$	1983	13
Thermophilic bacteria	$T_{min} > 30°C$, $T_{opt} > 55°C$, $T_{max} > 65°C$		
Extremely thermophilic bacteria	$T_{min} > 40°C$, $T_{opt} > 65°C$, $T_{max} > 75°C$		
Thermotolerant bacteria	$T_{opt} \leq 45°C$, $T_{max} > 45°C$	1984	14
Thermophilic bacteria	$T_{min} > 25°C$, $T_{opt} > 45°C$, $T_{max} > 50°C$		
Extremely thermophilic bacteria	$T_{min} > 37°C$, $T_{opt} \geq 65°C$, $T_{max} \geq 70°C$		
Barothermotolerant bacteria	$T_{opt} < 100°C$, $T_{max} > 100°C$		
Barothermophilic bacteria	$T_{opt} > 100°C$, $T_{max} > 100°C$		

Table 3

UPPER TEMPERATURE LIMITS FOR GROWTH OF VARIOUS MICROORGANISMS[a]

Microbial Group	Approximate Upper Temperature (°C)
Procaryotic Microorganisms	
blue-green algae	70-73
photosynthetic bacteria	70-73
chemolithotrophic bacteria	>90
heterotrophic bacteria	>90
Eucaryotic Microorganisms	
protozoa	56
algae	55-60
fungi	60-62
yeast	50

[a] adapted from (34)

Table 4

POTENTIAL MERITS OF THERMOPHILIC FERMENTATION

---

1. Fast growth rate.
2. Low cellular yield leading to high product/substrate ratios.
3. High metabolic activity leading to faster fermentation and increase in production rates.
4. Ferment wide range of substrates (sugars, starch, cellulose, hemicellulose).
5. pH and temperature can generally be varied over wide ranges, so the microorganism has the ability to withstand heat and acid shocks.
6. Many potential advantages of thermophilic enzymes.
7. Minimizes contamination risks (especially of pathogens).
8. Low oxygen solubility favours anaerobic conditions.
9. Reduces energy costs, easier and cheaper to heat fermentors (if necessary) than to cool them.
10. Cheaper distillation costs.
11. Facilitates continuous ethanol removal from ongoing fermentations (novel systems).
12. Since substrates are more soluble, enables use of higher substrate concentrations or substrates with lower solubility.
13. Less energy needed for mixing to maintain homogenous conditions due to reduced viscosity.
14. Facilitates removal of volatile inhibitors.
15. Reduced energy requirements for drying microorganisms for SCP.
16. Host vector systems safer from standpoint of biohazard.

Table 5

POTENTIAL LIMITATIONS OF THERMOPHILIC FERMENTATION

1. Lower ethanol tolerance of microorganisms, hence lower ethanol yield and/or productivity.
2. Lower substrate tolerance of microorganisms.
3. Formation of mixed by-products - acetic acid, lactic acid, etc.
4. Limited knowledge about physiology and biochemistry leads to further problems in scale-up.
5. Low oxygen solubility may hinder aerobic cultivation.
6. Extensive media evaporation.
7. Greater stress on biotechnological hardware (leaching of inhibitory trace metals from fermentation vessels, deterioration of biosensors).
8. Limited knowledge about distribution of thermophiles and their fitness into new environments.
9. Thermophiles may be less inherently stable.

Table 6

OTHER USES OF THERMOPHILIC MICROORGANISMS

Uses	References
Single-cell protein	86-91
Sewage and waste treatment, composting	56, 57, 92-97
Anaerobic digestion and methane production	79, 98-103
Extraction of ores and coal desulfurization	104-111
Enzymes	14, 82, 112-118
Chemical feedstocks (acetic acid)	59, 119-121
Dairy Industry	15, 122-124
Biotransformation	125-127
Antibiotics	128-131
H_2 production	132-134
Tobacco processing	22
Curing cocoa beans	135
Recovery of vegetable oils and fats	136
Degumming of silk	137

Table 7

ADVANTAGES OF THERMOSTABLE ENZYMES

1. Enhanced thermostability.
2. High catalytic activity so less enzyme is required.
3. Longer half-life so potential for enzyme reuse.
4. Greater resistance to denaturing agents.
5. Less contamination problems.
6. Broad substrate spectrum.
7. More tolerant to higher solute concentrations.
8. Since low activity at room temperature, unnecessary to cool enzymes during purification.
9. May further increase commercial use of immobilized enzymes.
10. May be more easily purified after cloning their genes in mesophiles and recovered by thermal separation.

Table 8

POSSIBLE PHYSIOLOGICAL BASIS OF THERMOSTABILITY OF CELLULAR COMPONENTS

Components of Protein Synthesis

- Higher GC content and T_m of nucleic acids
- DNA repair
- Rapid resynthesis (mRNA)
- Unique bases (tRNA-thiolated nucleotides, ribose methylations)
- Increase of thermostability of enzymes required for protein synthesis (tRNA synthetases, polymerases)
- Higher T_m of ribosome (especially 50S subunit)
- Interaction between ribosomes, ribosomal proteins, nucleic acids, and possibly membranes leading to conformational changes.

Proteins

- Amino acid composition and sequence (especially hydrophobic ones)
- Type of bonding (hydrophobic, ionic, hydrogen) directs protein folding and conformations
- Association with membranes
- Rapid protein resynthesis
- Activation or denaturation energy required
- Ionic strength
- Presence of cations (Ca^{+2}, Mg^{+2}, K^+, NH_4^+)
- Heat shock proteins
- Glycosylated proteins
- Salt bridges and aggregation
- Unique stabilizing factors
  - polyamines
  - polyols (glycerol)
  - trehalose.

Membranes and Lipids

- Composition of phospholipids (cardiolipin) and class of neutral lipids
- Shift in fatty acids with higher T_m (increase chain length, degree of branching, molecular geometry)
- Increase degree of saturation of fatty acids and/or sterols for more rigid membrane
- Unique lipids
- Increase protein/lipid ratio
- Reversible gel to liquid - crystalline phase transition
- Interaction of membrane proteins and lipids.

Table 9

LIQUID FUEL CHARACTERISTICS[a]

	Characteristics		
	Formula	Boiling Point (°C)	Lower Heating Value [b] (MJ/kg)
Fuels			
Gasoline	C_4-C_{12}*	32-210	44.5
Diesel	C_{14}-C_{20}*	204-343	43.0
Methanol	CH_3OH	65	19.6
Ethanol	CH_3CH_2OH	78	26.9
Butanol	$CH_3CH_2CH_2CH_2OH$	118	33.1

* hydrocarbons

[a] Adapted from (341)

[b] Lower heating value = heat of combustion at 25°C and constant pressure to form H_2O and CO_2

Table 10

POTENTIAL RAW MATERIALS FOR FERMENTATION ETHANOL

Non-Starch Sugar Sources	Starch Sources	Cellulose Sources
sugar cane (molasses)	corn	agricultural residues
sugar beet	rye	lumber mill residues (saw dust, wood chips)
sugar millet	rice	pulp and paper mill residues (SSL)
sweet sorghum	oats	forest residues
nipa palm	barley	municipal wastes (sewage, waste paper)
fodder beet	wheat	straw
fruit, juices	grain sorghum	bamboo
whey	cassava	cotton
Jerusalem artichoke (inulin)	potatoes	bagasse
cannery wastes	manioc	grasses
	tapioca	

Table 11

COMPARISON OF DESIRABLE FERMENTATION FEATURES OF YEASTS, *Zymomonas mobilis*, AND THERMOPHILIC BACTERIA

Desirable Features	Yeasts	*Zymomonas mobilis*	Thermophilic Bacteria
broad range of substrates (direct cellulose and hemicellulose fermentation)	-	-	+
high metabolic rate	+	+	+
low cellular yield	-	+	+
high substrate tolerance	+	+	-
high ethanol concentration	+	+	-
high ethanol tolerance	+	+	-
high ethanol productivity rate	+	+	-
few by-products	+	+	-
low energy costs (product recovery, cooling costs, mixing)	-	-	+
wide range of pH and temperature tolerance	-	-	+
easy to maintain sterile conditions	+	-	+
simple process technology available for scale-up	+	-	-

Table 12

MESOPHILIC BACTERIA WITH ETHANOL AS THE MAIN FERMENTATION PRODUCT[a]

Organism	Stoichiometry $\left(\frac{\text{moles ethanol}}{\text{moles glucose}}\right)$	Ref.
Clostridium sordelli	1.7	407
Zymomonas mobilis	1.9	408
Spirochaeta aurantia	1.5	409
S. stenostrepta	0.84	409
S. litoralis	1.1	409
Erwinia amylovora	1.2	410
Leuconostoc mesenteroides	1.1	407
Streptococcus lactis	1.0	122
Sarcina ventriculi	1.0	407

[a] adapted from (11)

Table 13

SUBSTRATE UTILIZATION AND PRODUCT FORMATION OF THERMOPHILIC ETHANOLOGENS

Substrates	*Clostridium thermohydrosulfuricum*	*Clostridium thermosaccharolyticum*	*Thermoanaerobium brockii.*	*Thermoanaerobacter ethanolicus*
Polymers				
xylan	+	+	+	+
starch	+	+	+	+
pectin	+			+
inulin	+			+
dextrin	+	+		+
Sugars				
glucose	+	+	+	+
cellobiose	+	+	+	+
xylose	+	+		+
maltose	+	+	+	+
fructose	+	+		+
mannose	+	+		+
sucrose	+	+	+	+
ribose	+			+
lactose	+	+	+	+
galactose	+	+		+
raffinose	+	+		
xylulose				+
arabinose	+	+	+	
melibiose		+		+
trehalose	+	+		
Others				
acetate		+		
pyruvate	+	+	+	+
mannitol	+			+
Products				
ethanol	+	+	+	+
acetic acid	+	+	+	+
lactic acid	+	+	+	+
CO_2/H_2	+	+	+	+
butyrate		+		

Table 14

PROPERTIES OF ANAEROBIC THERMOPHILIC ETHANOLOGENS

Organism	Stoichiometry $\frac{\text{(moles ethanol)}}{\text{(moles glucose)}}$	Doubling Time (Min)	Temperature Range (°C)			pH Range
			T_{min}	T_{opt}	T_{max}	
Clostridium thermocellum	1.0	420	45	55–62	65	5–7.5
C. thermosaccharolyticum	1.1	~75	37	58–64	70	4.8–7.6
Thermoanaerobium brockii	1	60	>35	65–70	<85	5.5–9.5
C. thermohydrosulfuricum	1.95	75	37	65–70	78	4.8–9.7
Thermoanaerobacter ethanolicus	1.95	135	37	69	78	4.5–9.8

Table 15

LIST OF YEAST SPECIES WITH REPORTED T_{max} ≥45°C[a]

Species Name	~T_{max} (°C)	Ref
Candida albicans	46	65
C. blankii	47	65
C. cacao	46	65
C. freyschussii	47	65
C. krusei	45	65
C. langeroni	45	65
C. lusitaniae	45	65
C. macedoniensis	48	65
C. pseudotropicalis	47	65
C. scotti	46	563
C. sloofii	46	26
Candida sp. HT4	48	21
C. valida	46	563
C. vinaria	46	563
Endoblastomyces thermophilus	48	22
Hansenula angusta	48	564
H. polymorpha	50	565
Kluyveromyces cicerisporus	46	563
K. fragilis	45	566
K. marxianus	49	567
Saccharomyces cerevisiae	45	21
S. fragilis	46	568
S. marxianus	47	569
S. thermantitonum	50	163
Torulopsis bovina	45	65
T. glabrata	46	65
T. hensenii	45	21
T. molischiana	45	65

[a] Species where fermentation was reported at temperatures ≥45°C are not included in this table. Only species where growth or T_{max} was ≥45°C are listed.

Table 16

Kluyveromyces spp. CAPABLE OF FERMENTATION AT TEMPERATURES ≥40°C

Strain	Temp. (°C)	Time (~hrs)	Substrate [% Glucose (w/v)]	ETOH Concentration (% w/v)	ETOH Productivity (g/ℓ/h)	Ref.
K. fragilis CSIR Y326	40	9	4	NA	2.62	580
	45	9	4	NA	0.63	580
	50	9	4	NA	0.18	580
K. fragilis YKL1	40	78	14	4*	--	579
K. marxianus (natural isolate)	40	NA	15	7.05	--	567
K. marxianus NCYC 587	40	72	14.5	5.03*	--	579
K. marxianus YKL6	40	78	14.5	4.47*	--	579
K. marxianus NCYC 827	40	NA	14	3.67*	--	**
K. marxianus NCYC 151	40	NA	14	2.47*	--	**
K. marxianus NCYC 179	40	NA	14	0.32*	--	**
Kluyveromyces sp. YKL 10	40	78	14.6	5.19*	--	579

*Ethanol values were initially reported on a v/v basis and were subsequently converted to w/v by multiplying by the conversion factor (0.7983)

**Hacking - personal communication

NA = Not Available

Table 17

Candida spp. CAPABLE OF FERMENTATION AT TEMPERATURES ≥40°C

Strain	Temp. (°C)	Time (hrs)	Substrate	ETOH Concentration (% w/v)	Ref.
Candida acidothermophilum	40	14	10% pretreated rice straw	2.30	585
C. brassicae IFO 1664	40	24	cellulose - SSF	0.24	586
	45	48	cellulose - SSF	0.19	586
C. lusitaniae Y-5394	41	NA	10% cellobiose	4.57	587
C. obtusa ATCC 24009	40	NA	14% (w/v) glucose	1.76*	**
C. pseudotropicalis YCa9	40	63	14.2% (w/v) glucose	6.87*	579
	45	NA	14% (w/v) glucose	3.99*	**
C. pseudotropicalis NCYC 143	40	NA	14% (w/v) glucose	3.75*	**
C. tropicalis NCYC 405	40	NA	14% (w/v) glucose	3.43*	**
C. utilis YCa25	40	62	14.1% (w/v) glucose	3.43*	579
Candida sp. HT4	45	48	10% (w/v) glucose	4.32	21
	50	6	4.5% (w/v) glucose	2.00	21
Candida sp. HT1	45	48	10% (w/v) glucose	4.07	21
Candida sp. HT2	45	48	10% (w/v) glucose	0.39	21
Candida sp. HT3	45	48	10% (w/v) glucose	3.42	21
Candida sp. HT5	45	48	10% (w/v) glucose	1.98	21
Candida sp. HT6	45	48	10% (w/v) glucose	3.65	21
Candida sp. HT7	45	48	10% (w/v) glucose	4.09	21
Candida sp. HT8	45	48	10% (w/v) glucose	3.20	21

*Ethanol values were initially reported on a v/v basis and were subsequently converted to w/v by multiplying by the conversion factor (0.7983).

**Hacking - personal communication

NA = Not Available

Table 18

Saccharomyces cerevisiae STRAINS CAPABLE OF FERMENTATION AT TEMPERATURES ≥40°C[a]

Strain	Temp (°C)	Time (hr)	Substrate	ETOH Concentration (% w/v)	Ref.
Saccharomyces cerevisiae ATCC 4132	40	NA	cellulose-SSF	4.39	588
	40	24	cellulose-SSF	0.24	586
	45	24	cellolose-SSF	0.06	586
S. cerevisiae ATCC 4126	40		14% (w/v) glucose	0.24*	**
	40	24	cellulose-SSF	0.21	586
	45	24	cellulose-SSF	0.06	586
S. cerevisiae ATCC 24858	40	24	cellulose-SSF	0.20	586
	45	24	cellulose-SSF	0.04	586
S. cerevisiae var. ellipsoides	40	24	cellulose-SSF	0.21	586
	45	24	cellulose-SSF	0.03	586
S. cerevisiae YSa86	40	63	13.5% (w/v) glucose	6.38*	579
S. cerevisiae NCYC 1380	40	NA	14% (w/v) glucose	3.59*	**
S. cerevisiae CBS 2970	40	NA	14% (w/v) glucose	2.71*	**
S. cerevisiae NCYC 447	40	NA	14% (w/v) glucose	2.23*	**
S. cerevisiae CBS 2966	40	NA	14% (w/v) glucose	2.00*	**
S. cerevisiae NCYC 713	40	NA	14% (w/v) glucose	1.27*	**
S. cerevisiae ATCC 26603	40	NA	14% (w/v) glucose	0.72*	**
S. cerevisiae 1200	40	NA	molasses	7.03*	590
S. cerevisiae NP3	40	NA	molasses	7.58*	590
S. cerevisiae N1	40	NA	35% (w/v) glucose	9.6	591

continued.....

Table 18 Cont'd

Saccharomyces cerevisiae STRAINS CAPABLE OF FERMENTATION AT TEMPERATURES ≥40°C

Strain	Temp (°C)	Time (hr)	Substrate	ETOH Concentration (% w/v)	Ref.
S. cerevisiae TJ 1	40	NA	35% (w/v) glucose	7.0	591
S. cerevisiae AM 12	40	NA	35% (w/v) glucose	12.4	591
S. cerevisiae Y2	40	NA	molasses	9.63	592
S. cerevisiae Y3	40	NA	molasses	9.40	592
S. cerevisiae Y16	40	NA	molasses	9.48	592
S. cerevisiae Y17	40	NA	molasses	10.16	592
S. cerevisiae Y18	40	NA	molasses	9.63	592
S. cerevisiae Y21	40	NA	molasses	9.36	592
S. cerevisiae Y22	40	NA	molasses	9.33	592
S. cerevisiae Y23	40	NA	molasses	9.72	592
S. cerevisiae Y24	40	NA	molasses	10.27	592
S. cerevisiae Y26	40	NA	molasses	10.19	592
S. cerevisiae Y29	40	NA	molasses	9.68	592

NA = Not Available

*Ethanol values were initially reported on a v/v basis and were subsequently converted to w/v by multiplying by the conversion factor (0.7983).

**Hacking - personal communication

[a] Other strains of *Saccharomyces cerevisiae* where fermentation was reported at temperatures ≥40°C are listed in Table 25.

Table 19

Saccharomyces spp. CAPABLE OF FERMENTATION AT TEMPERATURES ≥40°C

Strain	Temp (°C)	Time (hr)	Substrate	ETOH Concentration (% w/v)	Ref.
Saccharomyces appiculatus	47	NA	---	NA	593
S. chevalier	41	NA	40% (w/v) sorghum juice	NA	594
S. diastaticus	43	NA	40% (w/v) sorghum juice	NA	594
S. diastaticus 1384	40	24	20% (w/v) glucose	6.0	595
Saccharomyces sp Strain 1400 (fusion product)	40	24	20% (w/v) glucose	7.0	595
S. uvarum (carlsbergensis)	41	NA	40% (w/v) sorghum juice	NA	594
S. uvarum (carlsbergensis) IAM 4787	40	NA	cellulose-SSF	3.86	588
	40	24	cellulose-SSF	0.19	586
	45	24	cellulose-SSF	[illegible].8	586
S. uvarum (carlsbergensis) ATCC 26602	40	NA	14% (w/v) glucose	0.16*	**
	43	NA	10% (w/v) glucose	3.80	411
S. uvarum (carlsbergensis) YSa85	40	62	14.5% (w/v) glucose	6.55*	579
S. uvarum (carlsbergensis) inulyticus	40	36-48	sucrose (stepwise addition)	7.82*	596

* Ethanol values were initially reported on a v/v basis and were subsequently converted to w/v by multiplying by the conversion factor (0.7983).

** Hacking - personal communication

NA = Not Available

Table 20

OTHER YEAST SPECIES CAPABLE OF FERMENTATION AT TEMPERATURES ≥40°C

Strain	Temp (°C)	Time (hr)	Substrate	ETOH Concentration (% w/v)	Ref.
Hansenula polymorpha ATCC 4516	45	48	10% (w/v) glucose	1.80	21
H. polymorpha ATCC 14754	45	48	10% (w/v) glucose	0.46	21
H. subpelliculosa NCYC 558	40	NA	14% (w/v) glucose	2.39*	**
Lambanog isolate	40-42	72	molasses***	3.87	601
	45-47	72	molasses***	3.42	601
	50-52	72	molasses***	2.86	601
Rice cake yeast	40	72	pineapple wastes	11.00	599
Schizosaccharomyces pombe YSC 3	40		14% (w/v) glucose	2.15*	**

NA = Not Available

* Ethanol values were initially reported on a v/v basis and were subsequently converted to w/v by multiplying by the conversion factor (.7983).

** Hacking - personal communication

*** Molasses diluted to contain 10% reducing sugar

Table 21

THERMOTOLERANCE OF YEAST GENERA TESTED[a]

Genus	Number of Species Total Tested at 37°C	Above 50% Ethanol Yield at 37°C	Above 50% Ethanol Yield at 40°C	Growth and Ethanol Production at 45°C
Candida	15	5	4	1
Hansenula	7	1	0	0
Kluyveromyces	12	8	5	5
Pichia	4	0	0	0
Saccharomyces	14	13	3	0
Schizosaccharomyces	2	1	0	0
Torulopsis	1	0	0	0
Total	55	28	12	6

[a] Hacking - personal communication

Table 22

ETHANOL CONCENTRATIONS OF THERMOTOLERANT STRAINS IN FLASKS AND/OR FERMENTORS[a]

Strain	Ethanol Yield (% v/v) 40°C Shake Flask	40°C 5 ℓ Fermentors (time in hours)	45°C Shake Flask
Candida pseudotropicalis NCYC 143	4.7	ND	0*
C. pseudotropicalis YCa9	4.9	8.6 (63 h)	5.0
C. tropicalis NCYC 405	4.3	ND	0*
C. utilis YCa25	5.3	4.3 (62 h)	0*
Kluyveromyces sp. YKL10	6.1	6.5 (78 h)	1.4
K. fragilis YKL1	4.9	5.0 (78 h)	3.1
K. marxianus YKL6	4.8	5.6 (78 h)	4.3
K. marxianus NCYC 587	6.3	6.3 (72 h)	1.7
K. marxianus NCYC 827	4.6	ND	3.8
Saccharomyces cerevisiae NCYC 1380	4.5	ND	0*
S. cerevisiae YSa86	5.4	8.0 (63 h)	0*
S. uvarum YSa85	5.6	8.2 (62 h)	0*

[a] adapted from (579, Hacking - personal communication)

ND = Not Done

* denotes no growth

Table 23

EFFECT OF TEMPERATURE ON THE KINETIC PARAMETERS OF *Kluyveromyces marxianus*[a]

Parameters	Temperature (°C)				
	30	40	43	45	48
max. specific growth rate (hr^{-1})	0.29	0.32	0.33	0.38	0.31
max. specific glucose consumption rate	3.67	7.11	8.92	9.05	11.81
max. ethanol production rate (g/g/h)	1.66	3.55	4.31	4.15	3.98
% glucose utilized	92	99	93	76	56
ethanol yield (g/glucose used)	0.41	0.47	0.43	0.37	0.26

[a] adapted from (567)

Table 24

EFFECT OF TEMPERATURE ON THE KINETIC PARAMETERS OF *Saccharomyces uvarum (carlsbergensis)*[a]

Parameters	Temperature (°C)					
	25	30	33	37	40	43
max. specific growth rate (hr^{-1})	0.19	0.23	0.26	0.23	0.21	0.16
max. ethanol production rate (g/g/h)	0.94	1.15	1.33	1.44	1.44	1.43
ethanol yield (g/glucose)	0.377	0.377	0.377	0.377	0.377	0.377

[a] adapted from (597)

Table 25
MOLASSES FERMENTATION[a] OF VARIOUS YEAST STRAINS AT HIGH TEMPERATURE[b]

Strains	Molasses Concentration (°Brix)[c]	Ethanol Productivities (g/ℓ/h)	
		45°C	42°C
Saccharomyces cerevisiae CBS 1196	30	30.0	35.5
	40	14.5	30.0
	50	5.5	7.0
S. cerevisiae ATCC 4126	30	31.5	40.5
	40	29.5	30.0
	50	4.5	4.0
S. cerevisiae CBS 1235	30	16.5	41.0
	40	0.5	7.0
	50	0.5	1.0
S. cerevisiae CSB 1237	30	27.0	35.0
	40	23.5	27.0
	50	6.0	8.0
S. cerevisiae CBS 1242	40	40.5	41.0
	40	17.5	18.5
	50	5.0	4.5
S. cerevisiae CBS 2970	30	46.5	48.0
	40	21.0	24.5
	50	1.5	3.0
S. cerevisiae (fresh yeast SJAB)	30	53.0	33.0
	40	25.5	24.0
	50	15.5	13.5
Schizosaccharomyces pombe CBS 352	30	38.5	39.0
	40	41.5	37.0
	50	31.0	30.0
S. pombe CBS 357	30	35.0	27.5
	40	26.0	23.5
	50	22.0	23.0
S. pombe (Alfa-Laval)	30	13.0	25.0
	40	17.5	26.0
	50	19.0	23.5
Saccharomyces uvarum (carlsbergensis) ATCC 2345	30	4.0	7.5
	40	1.0	5.0
	50	0.5	0.5

[a] fermentations were carried out in 500 ml shake flasks. The yeast concentration in all runs was 5% wet weight.

[b] adapted from (598)

[c] 30°Brix = 33.61% (w/v)

Table 26

COMPARISON OF MONO- AND CO-CULTURE FERMENTATIONS OF CELLULOSICS BY *Clostridium thermocellum* AND *Clostridium thermohydrosulfuricum*[a]

Substrates	Culture	Products Formed (mM)		CMCase (U/ml)	Reducing Sugar mg/ml	Substrate Consumed (%)	Ethanol/Acetate Ratio (mole/mole)
		Ethanol	Acetate				
Aspen Wood (untreated)	mono-	2.2	6.4	—	—	—	0.34
	co-	8.4	9.3	—	—	—	0.90
MN300 Cellulose (0.7%)	mono-	31.2	22.5	2.66	1.28	60	1.39
	co-	88.9	4.2	0.79	0.08	100	21.1
Solka Floc (1.0%)	mono-	30.8	27.3	4.61	2.89	50	1.13
	co-	98.7	11.3	1.54	0.15	80	8.73
SO_2-Treated Aspen Wood	mono-	16.9	13.6	2.14	1.55	—	1.24
	co-	54.3	11.2	1.0	0.64	—	4.85
Steam Exploded Wood (1.0%)	mono-	14.9	15.2	2.97	1.58	—	0.98
	co-	63.9	6.6	0.72	0.12	—	9.68

[a] adapted from (456)

Table 27

COMPARISON OF DIFFERENT STRAINS OF *Clostridium thermosaccharolyticum* WITH RESPECT TO PRODUCT FORMATION[a]

Strain Designation	HG-3	HG-4	HG-6
Fermentation Time (hours)	85	52	50
Ethanol (% w/v)	1.42	2.67	3.78
Acetic Acid (% w/v)	1.00	0.63	0.95
Lactic Acid (% w/v)	1.57	0.25	0.30
Xylose Consumed (% w/v)	6.78	7.56	9.44
Yield of Ethanol (g ETOH/g xylose)	0.21	0.35	0.40

[a] adapted from (693)

Table 28

EFFECT OF XYLOSE AND CELLOBIOSE ON FERMENTATIONS[a] OF STEAM EXPLODED WOOD (SEW) USING *Clostridium thermocellum* JW20 ALONE AND IN CO-CULTURE WITH *Thermoanaerobacter ethanolicus* JW200[b]

Strain	SEW (mg/10 ml)	Sugars Supplemented (mg/10 ml)		Ethanol (mM)
		Xylose	Cellobiose	
C. thermocellum JW20	100	--	--	11
C. thermocellum JW20 plus *T. ethanolicus* JW200	100	--	--	31
C. thermocellum JW20 plus *T. ethanolicus* JW200	100*	--	--	44
C. thermocellum JW20	100	5	--	5
C. thermocellum JW20	100	--	5	8
C. thermocellum JW20	100	5	5	5
C. thermocellum JW20 plus *T. ethanolicus* JW200	100	5	5	57
C. thermocellum JW20 plus *T. ethanolicus* JW200	---	5	5	13

[a] Fermentations were carried out for 14 days at 60°C and pH 6.2 in 10 ml cultures containing 100 mg of SEW and/or the indicated supplemented sugars.

[b] Adapted from (450)

* pH was 7.5

Table 29

COMPARISON OF APPROXIMATE SPECIFIC ETHANOL PRODUCTION RATES (q_p) AND PRODUCT YIELDS ($Y_{p/s}$)[a]

Organisms	q_p [b]	$Y_{p/s}$ [c]
Clostridium thermocellum	0.23	0.8
C. thermohydrosulfuricum	0.8	0.5-1.5
Thermoanaerobium brockii	0.33-0.7	0.7-2.7
Thermobacteroides acetoethylicus	0.2-5[d]	1.5
Thermoanaerobacter ethanolicus	1.4[d]	1.9
Zymomonas mobilis	3-5	1.9
Saccharomyces cerevisiae	0.5-1	1.5

[a] adapted from (13)

[b] Units of q_p : g ethanol/g biomass/h

[c] Units of $Y_{p/s}$: mol ethanol formed/mol substrate

[d] Extrapolated (see ref. 13, 82)

Table 30

EFFECT OF HIGH TEMPERATURE ON PHYSICAL AND CHEMICAL PROPERTIES OF WATER[a]

Properties that Increase	Properties that Decrease
volume	density
vapor pressure	viscosity
diffusion	surface tension
ionization	dielectric constant
solubility of most compounds	heat capacity
	compressibility
	refractive index
	pH
	solubility of gases

[a] adapted from (30)

Table 31

METABOLIC REGULATION IN THERMOPHILIC ETHANOLOGENS[a]

Regulatory Properties	Metabolic Influence
1. Regulatory Enzymes	
a) NAD(P) dependent ADH	a) ethanol yields related to ethanol inhibition and NAD
b) FDP-activated LDH	b) lactate yields related to intracellular levels of FDP
c) ferredoxin - NAD(P) oxidoreductases and hydrogenases	c) controls ratio of (ethanol + lactate)/(acetate + H_2)
2. Interactions with chemical electron acceptors or donors	
a) acceptors - ketones, sulfite, thiosulfate	a) increases growth rate and cell yield; acetate is the main product
b) donors - H_2	b) decreases growth rate and final cell yield; ratio of (ethanol + lactate)/(acetate + H_2) is increased
3. Interactions between different species	
a) cellulolytic and saccharolytic ethanol producers	a) increases utilization of cellulose and hemicellulose components; increases ethanol yield and productivity rate
b) saccharolytic and H_2 oxidizing species (biological electron acceptor)	b) increases acetate and decreases ethanol yield

[a] adapted from (48)

Table 32

EFFECT OF H_2 ON GLUCOSE[a] FERMENTATION PRODUCTS
OF *Thermoanaerobium brockii*[b]

	Additions							
Products Formed	None	0.3 atm H_2	0.5 atm H_2	1 atm H_2	Acetone[c]	0.3 atm H_2 + Acetone	0.5 atm H_2 + Acetone	1 atm H_2 Acetone
H_2 Production/ Consumption (μmoles)	44	11	ND	ND	6	−77	−118	−153
Isopropanol (μmoles)	ND	ND	ND	ND	495	508	552	615
L-lactate (μmoles)	235	205	250	50	112	135	116	164
Ethanol (μmoles)	166	174	150	20	23	21	20	18
Acetate (μmoles)	38	2	16	19	316	300	287	198

ND = Not Detected

[a] 250 μmol glucose

[b] adapted from (48)

[c] 700 μmol acetone

Table 33

RELATION OF ENERGY SOURCES TO GROWTH, FRUCTOSE DIPHOSPHATE AND FERMENTATION PRODUCT YIELDS OF *Thermoanaerobium brockii*[a]

	Additions				
	None	Glucose	Starch	Acetone	Glucose & Acetone
Growth (O.D.$_{540}$)	0.15	1.3	1.0	0.40	2.3
[FDP] (μmol/mg cells)	--	52	2	---	21
H_2 (μmol/tube)	8	11	20	3	4
Ethanol (μmol/tube)	33	110	215	3	15
Acetate (μmol/tube)	11	15	29	90	262
Lactate (μmol/tube)	16	162	81	15	20
Ethanol/Lactate	2.0	0.70	2.65	0.2	0.75

[a] adapted from (27)

Table 34

ETHANOL FORMATION RATES WITH RESTING CELLS OF *Thermoanaerobacter ethanolicus* JW200 AND ITS MUTANTS JW200 Fe(4) AND JW200L-LARGE[a]

Strain	Substrate (% w/v)	Ethanol Productivity (mmole/ℓ/h)
JW200 (wild type)	0.8% glucose	30
	6.0% glucose	22
	0.8% xylose	22
	3.5% xylose	25
JW200 Fe(4)	0.8% glucose	45
	0.8% xylose	22
	3.5% xylose	27
JW200L-Large	0.8% starch	45
	0.8% glucose	60

[a] adapted from (470)

Table 35

EFFECT OF SUPPLEMENTATION[a] ON ETHANOL YIELDS
OF *Saccharomyces cerevisiae* STRAINS 1200 AND NP-3[b]

Medium	Final Ethanol Yields (% v/v)			
	30°C		40°C	
	1200	NP-3	1200	NP-3
molasses medium	18.6	17.4	8.8	9.5
molasses medium and grated carrot	22.0	18.9	13.0	11.4
molasses medium	ND	ND	6.7*	8.3*
molasses medium and lipid protein complex	ND	ND	9.0*	9.4*

[a] 3 separate experiments were performed. The inoculum size was 3.10^8 cells/ml, unless otherwise indicated by *.

[b] adapted from (590)

ND - Not Done

* inoculum size was 1.10^8

Table 36

EFFECT OF LINSEED OIL AND MIXED FATTY ACIDS DERIVED FROM LINSEED OIL ON MOLASSES FERMENTATION[a] BY *Saccharomyces cerevisiae* AT 30 AND 40°C[b]

	Time (hr)	Control		Linseed Oil		Fatty Acids	
		30°C	40°C	30°C	40°C	30°C	40°C
Total Reducing Sugars (g/100 ml)	0	13.09	13.15	12.96	13.22	12.77	13.15
	24	6.42	9.22	5.65	6.63	5.61	6.06
	48	2.66	5.95	1.58	3.92	1.20	3.53
	72	1.15	3.31	0.98	3.03	0.53	1.82
Ethanol (% w/v)	24	2.5	1.6	3.0	2.8	3.4	2.9
	48	4.4	2.9	4.6	3.9	5.3	3.9
	72	4.9	3.5	5.0	4.3	5.9	4.4
Fermentation Efficiency (%)	24	72.3	78.2	89.2	85.9	92.1	81.6
	48	82.1	79.5	77.3	82.2	90.4	81.1
	72	79.6	71.3	80.3	75.7	94.7	76.1

[a] medium used 20° Brix molasses (≃21.6% w/v); concentration of supplements 0.5% (v/v)

[b] adapted from (904)

Table 37

POSSIBLE DETRIMENTAL EFFECTS OF ETHANOL ON MICROBIAL CELLS

1. Kills cells (decreases cell viability).
2. Inhibits growth rate.
3. Inhibits fermentation rate.
4. Inhibits transport and/or uptake (sugars, ammonium, amino acids).
5. Denatures enzymes (glycolytic).
6. Inhibits enzyme activity.
7. Acts as an uncoupler.
8. Alters membrane permeability and thus membrane protein-mediated phenomena.
9. Modifies lipids and changes lipid-protein interations.
10. Enhances thermal death (decreases T_{max}).
11. Increases T_{min} and decreases T_{opt}.
12. Causes mitochondrial damage (induces petites).
13. Interferes with cell division.

Table 38

HYPOTHETICAL MODEL TO ACCOUNT FOR INCREASED ETHANOL INTOLERANCE IN *Saccharomyces spp.* AT HIGHER TEMPERATURES AS DIRECTED BY MEMBRANE PERMEABILITY CHANGES[a]

Temperature (°C)	Ethanol Concentration (% w/v)	Physical Effect on Membrane	Cell Response to Membrane Alteration	Consequence for Cell
30	0	no significant effect on permeability	adjusts fluidity for proper functioning	survival
30	5	permeability decreases slightly due to ethanol	permeability increases slightly to counteract effect of ethanol	survival
30	10	permeability decreases significantly due to ethanol	cannot respond sufficiently - membrane too rigid	death
35	0	permeability increases slightly due to higher temperature	permeability decreases slightly to counteract effect of higher temperature	survival
35	3	permeability increases slightly but less than in the absence of ethanol	permeability decreases but less than in the absence of ethanol	survival
35	6	ethanol tightens membrane further	permeability decreases due to higher temperature - cells cannot sufficiently respond to added physical effect of ethanol - membrane too rigid	death
45	0	permeability increases significantly	excessive membrane tightening	death

---

[a] Assuming the yeast strain to have a T_{max} of 40, 35, and 30°C in the presence of 0, 4, and 8% (w/v) ethanol, respectively. Hypothetical model should account for the inability of this yeast to survive in 10% (w/v) ethanol at 30°C, 6% (w/v) ethanol at 35°C, and in the absence of ethanol at 45°C.

Table 39

POTENTIAL ADVANTAGES OF GAS STRIPPING ETHANOL PURIFIER

---

1. Drastically reduces distillation costs (10-20% ethanol in raw product).
2. Cleaner product, only volatiles are removed from broth, not solids.
3. No need for flocculent culture.
4. Since positive pressure in system, less chance of contamination.
5. Since heat dissipated in evaporation, no cooling required.
6. No mechanical mixing needed since CO_2 sparging keeps cells evenly dispersed.
7. Offers advantages of other continuous processes - no need for cleaning equipment, reduces need for cell growth, etc.
8. Probably lower capital costs.
9. No mechanical seals.
10. Continuous ethanol removal may lessen ethanol toxicity and allow for higher productivities.
11. Most suitable for thermotolerant and thermophilic anaerobic microorganisms (including *C. thermocellum*), therefore potential for mixed culture cellulose fermentations.
12. May be suitable for any fermentation product that is sufficiently volatile to be stripped (eg., acetone, butanol).

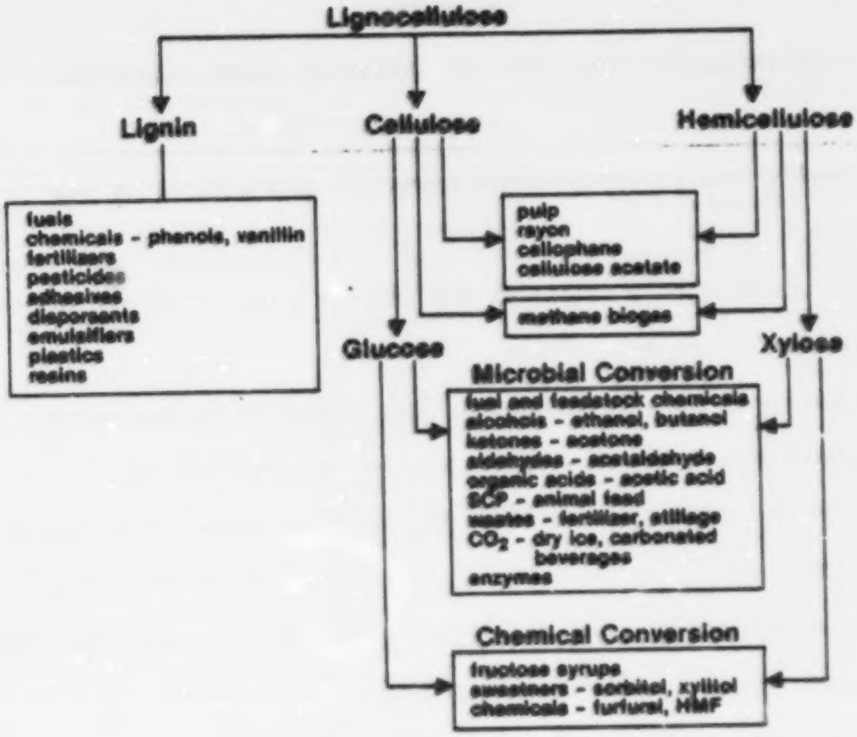


Figure 1. Potential by-products of cellulosic biomass.

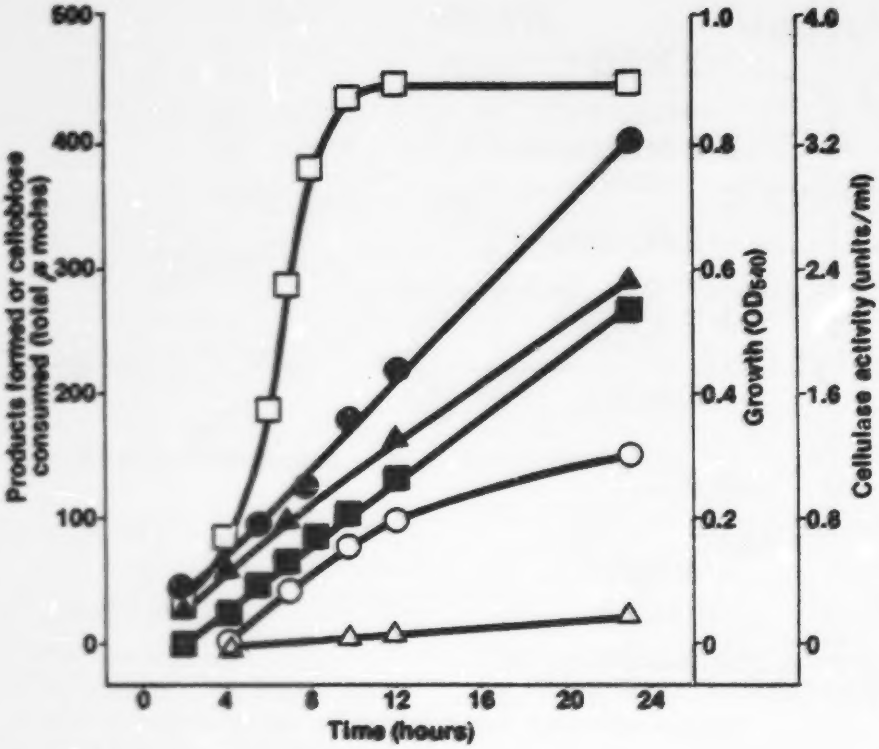
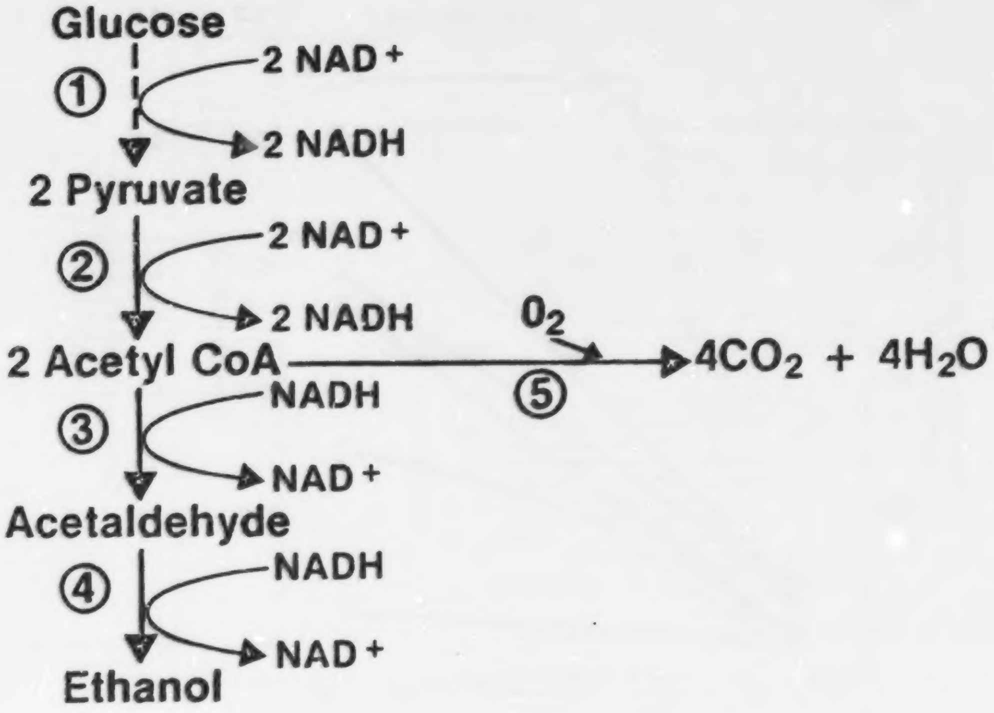


Figure 2. Fermentation characteristics of *Clostridium thermocellum* AS39 cultivated at 60°C in cellobiose medium. Lactate (△), cellobiose consumed (○), acetate (■), CMCase (▲), ethanol (●), growth (□).
Adapted from Lamed and Zeikus (461).



1. Glycolytic pathway
2. Pyruvate dehydrogenase
3. Acetaldehyde dehydrogenase
4. Alcohol dehydrogenase
5. TCA cycle

Figure 3. Aerobic pathway in *Bacillus stearothermophilus*. Adapted from Hartley and Payton (369).

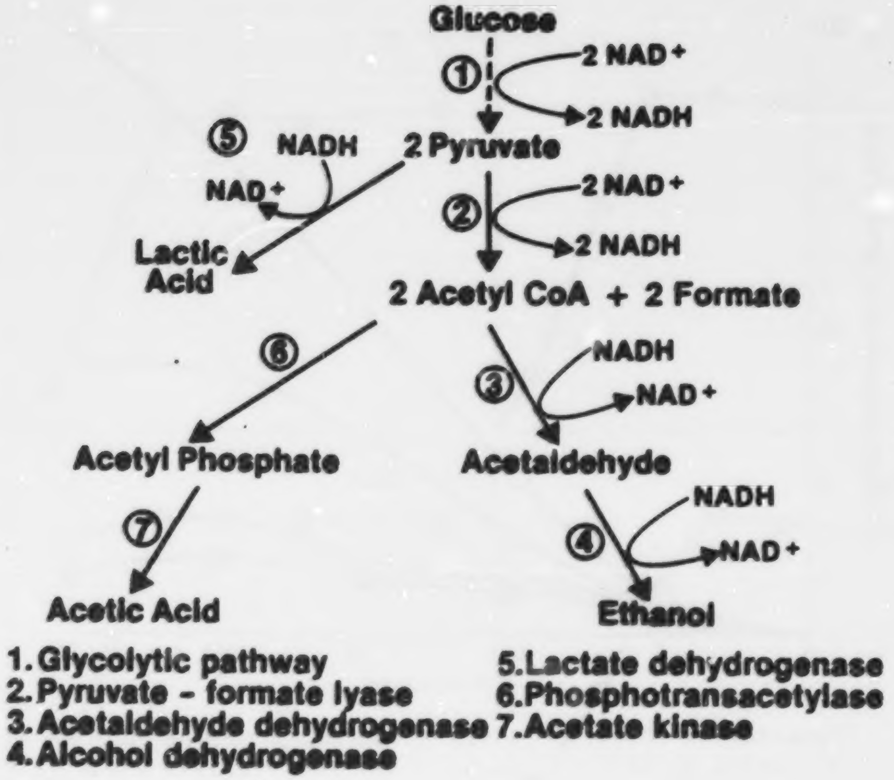


Figure 4. Hypothetical anaerobic pathway in *Bacillus stearothermophilus*. Adapted from Hartley and Payton (369).

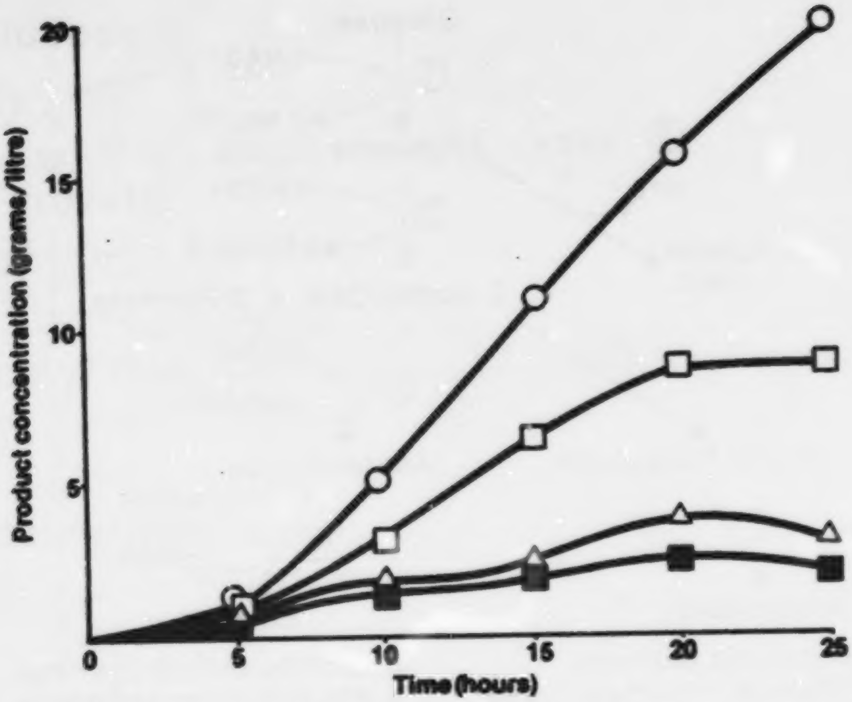


Figure 5. Fermentation characteristics of *Bacillus stearothermophilus* LLD-15 cultivated at 60°C in 5% (w/v) sucrose tryptone-yeast extract medium. Cell dry weight (■), acetate (△), formate (□), ethanol (○).
Adapted from Hartley and Payton (369).

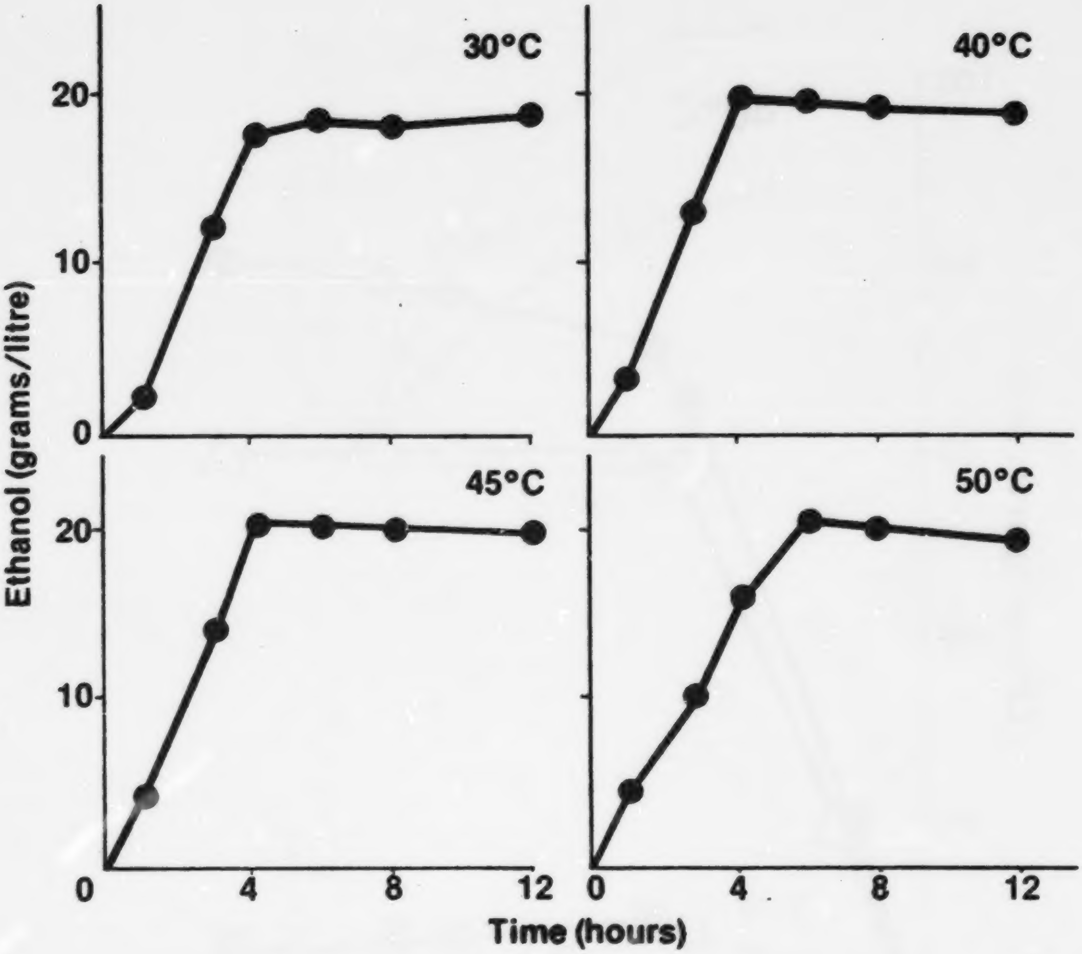


Figure 6. Effect of temperature on D-glucose (4.5% w/v) fermentation by *Candida sp.* HT4.
Adapted from McCracken and Gong (21).

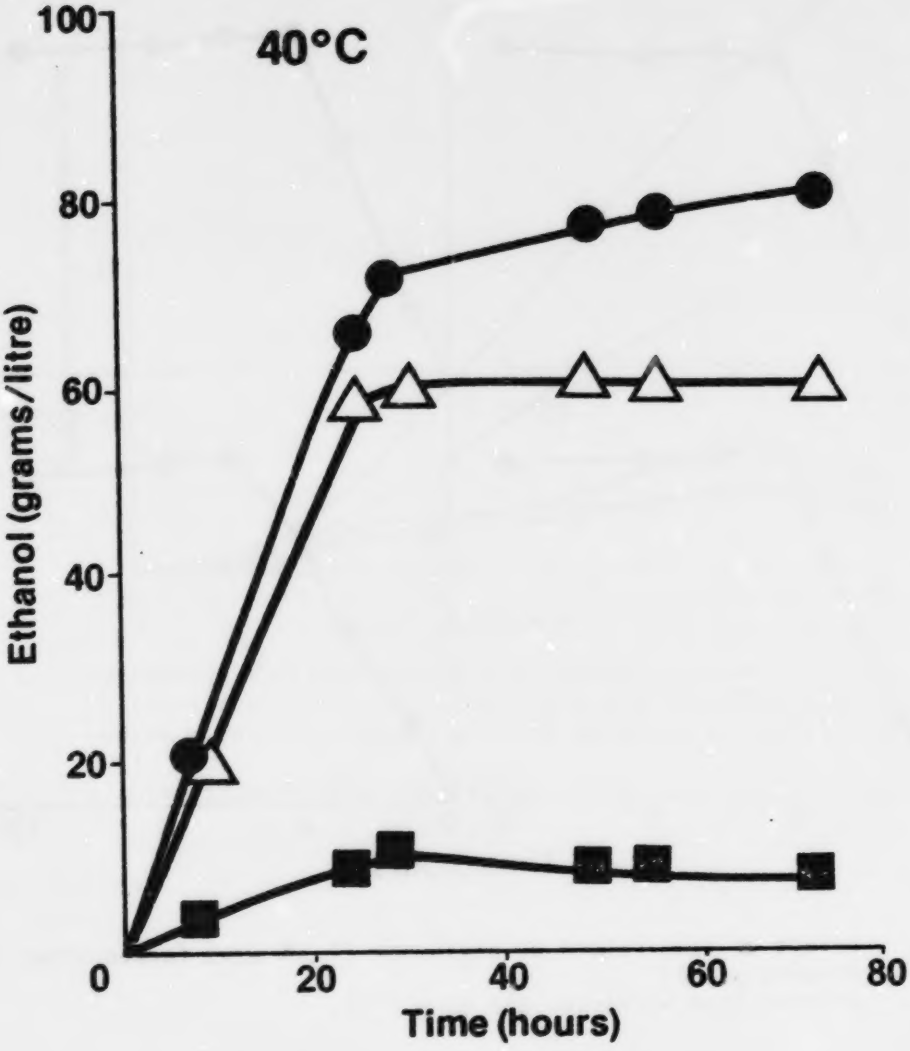


Figure 7. Ethanol production of *Saccharomyces spp.* at 40°C in 20% (w/v) glucose peptone-yeast extract medium. *Saccharomyces uvarum (carlsbergensis)* (strain 21) (■), *Saccharomyces diastaticus* (strain 1384) (△), fusion product (strain 1400) (●). Adapted from Stewart *et al.* (595).

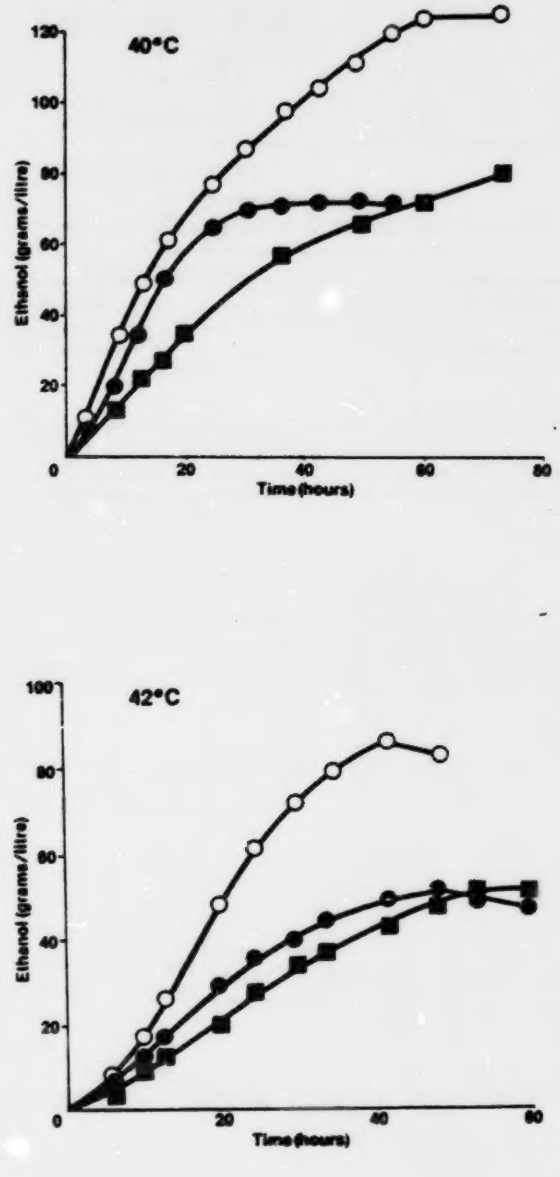


Figure 8. Ethanol production of *Saccharomyces cerevisiae* strains at 40°C and 42°C in 35% (w/v) glucose medium. Strain TJ1 (●), strain N1 (■), fusion product AM12 (○).
Adapted from Seki *et al.* (591).

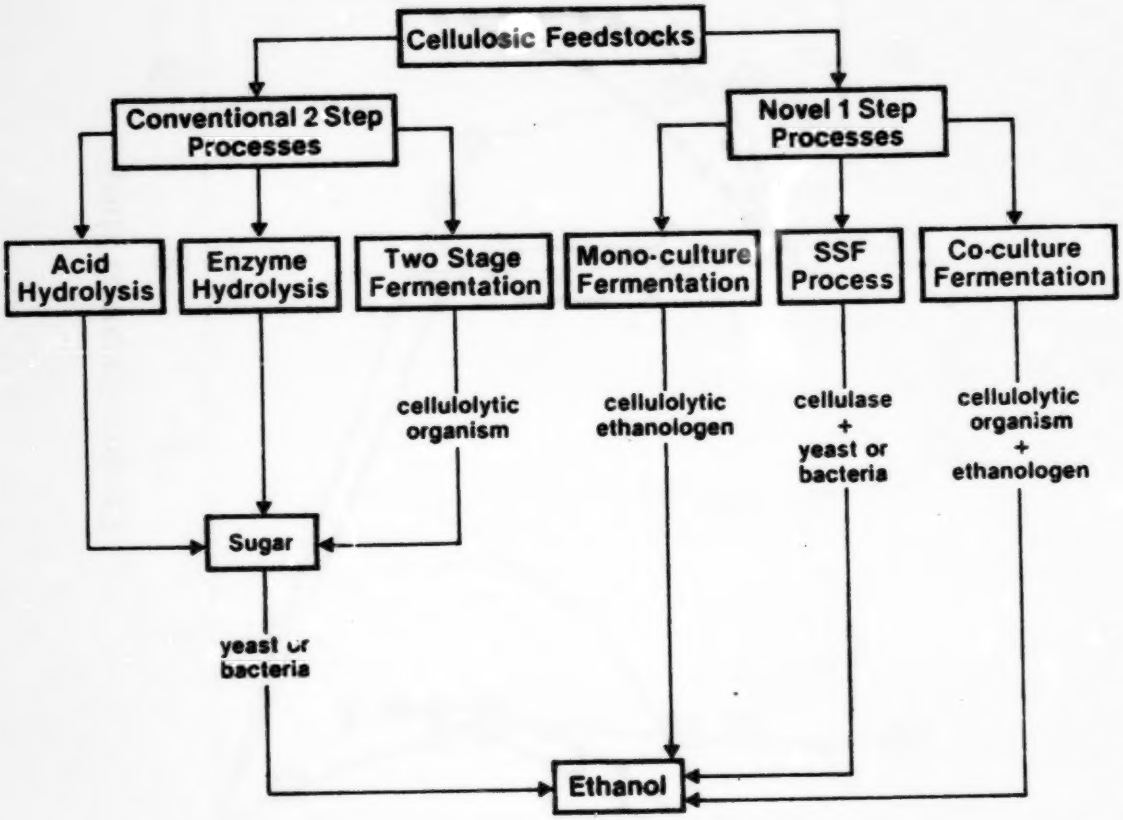
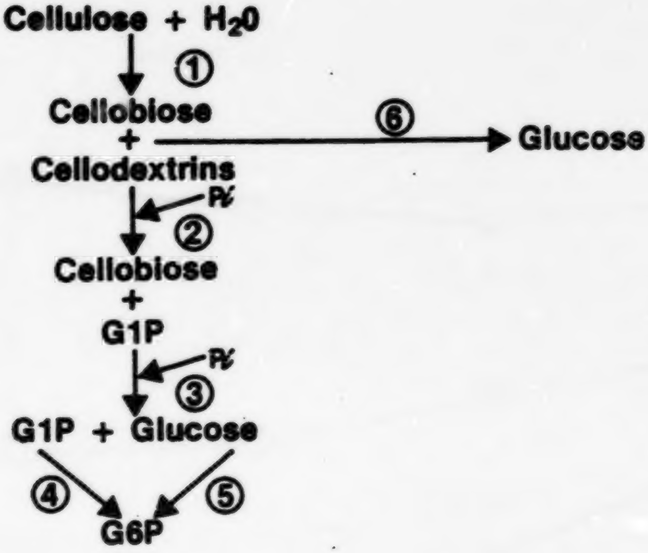


Figure 9. Major routes of ethanol production from cellulosics.



1. Exo- and endo-glucanase
2. Cellodextrin phosphorylase
3. Cellobiose phosphorylase
4. Phospho-glucomutase
5. Glucokinase
6. β-glucosidase

Figure 10. Hypothetical pathway for cellulose degradation by *Clostridium thermocellum*.

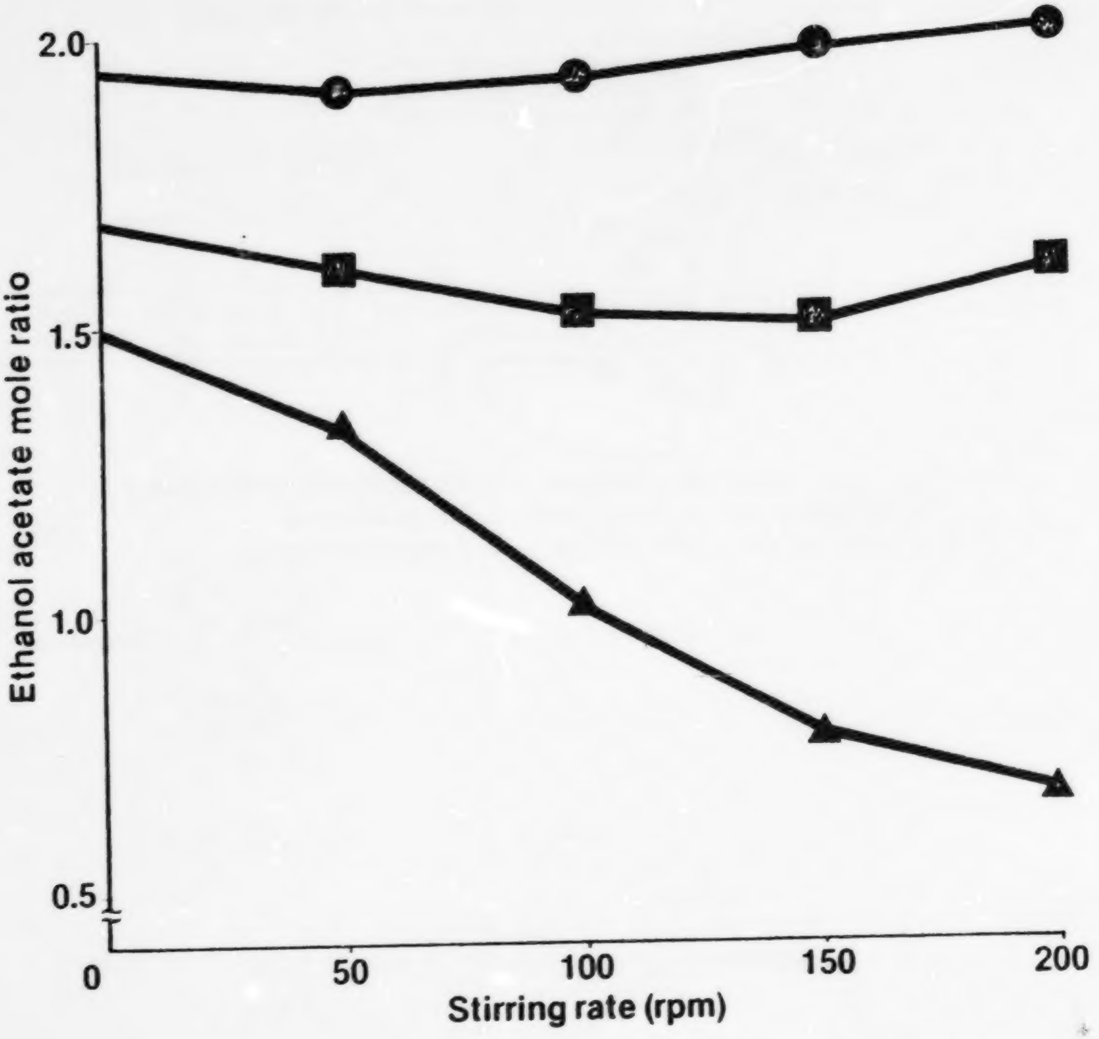


Figure 11. Effect of stirring and added H_2 on ethanol acetate mole ratio of *Clostridium thermocellum* YS grown on 2% (w/v) cellulose. No hydrogen (▲), 1 atm. H_2 (■), 2.5 atm. H_2 (●). Adapted from Su *et al.* (685).

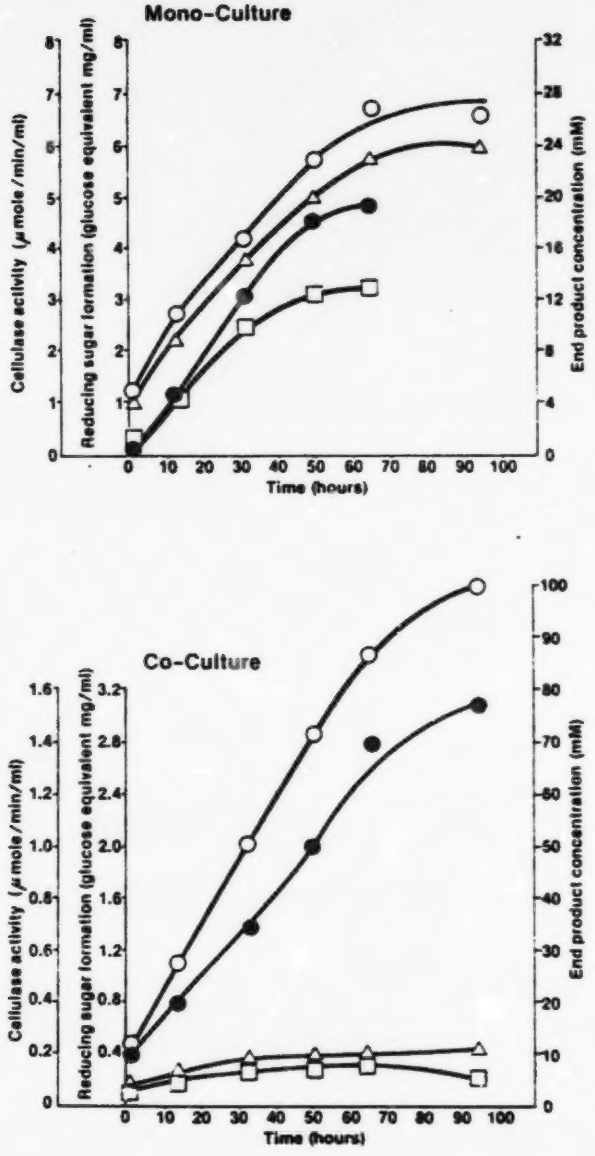


Figure 12. Cellulose fermentation of 1% (w/v) solka floc in mono-culture by *Clostridium thermocellum* LQR1 and in co-culture with *Clostridium thermohydrosulfuricum* 39E. Sugars (□), CMCase (●), acetate (△), ethanol (○).
Adapted from Ng *et al.* (456).

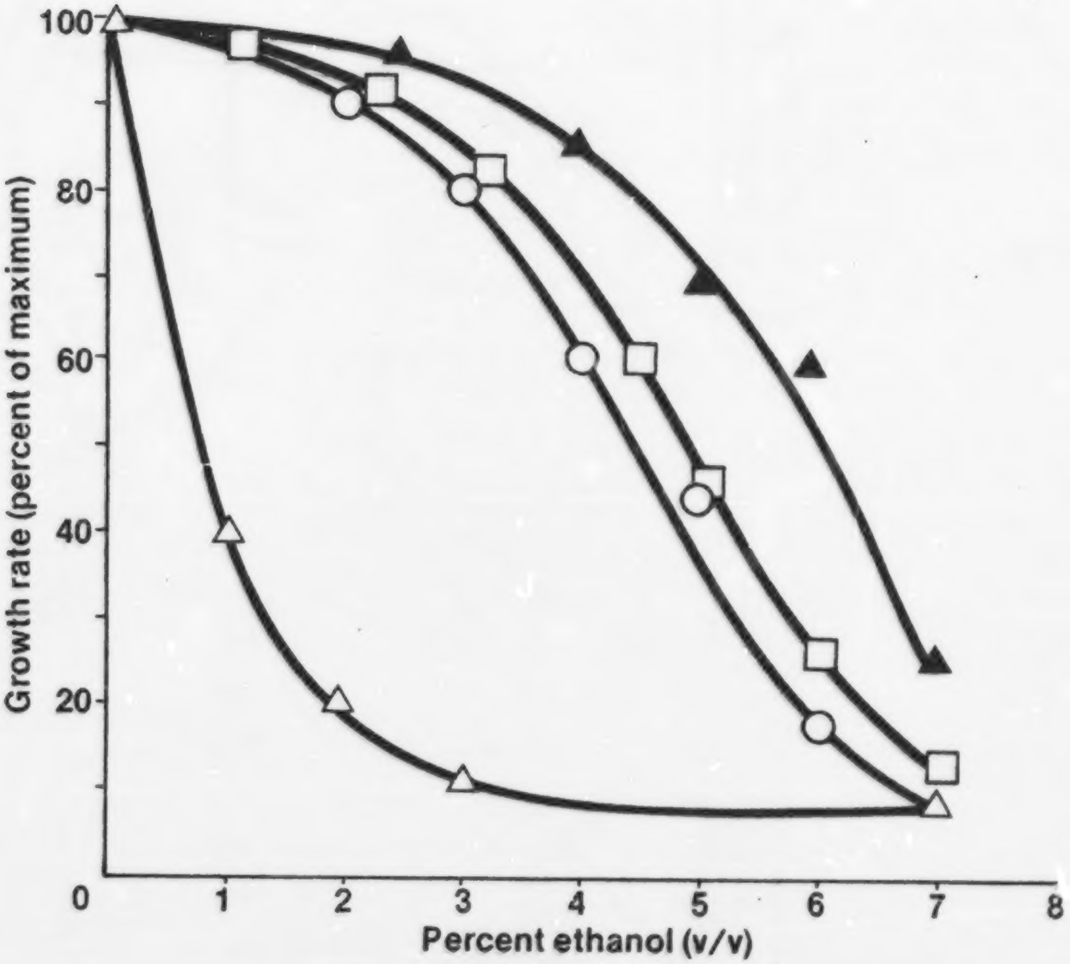


Figure 13. Growth rates of *Clostridium thermocellum* isolates in the presence of ethanol. Wild type ATCC 27405 (△), isolate S-4 (○), isolate S-6 (□), isolate S-7 (▲).
Adapted from Avgerinos *et al.* (439).

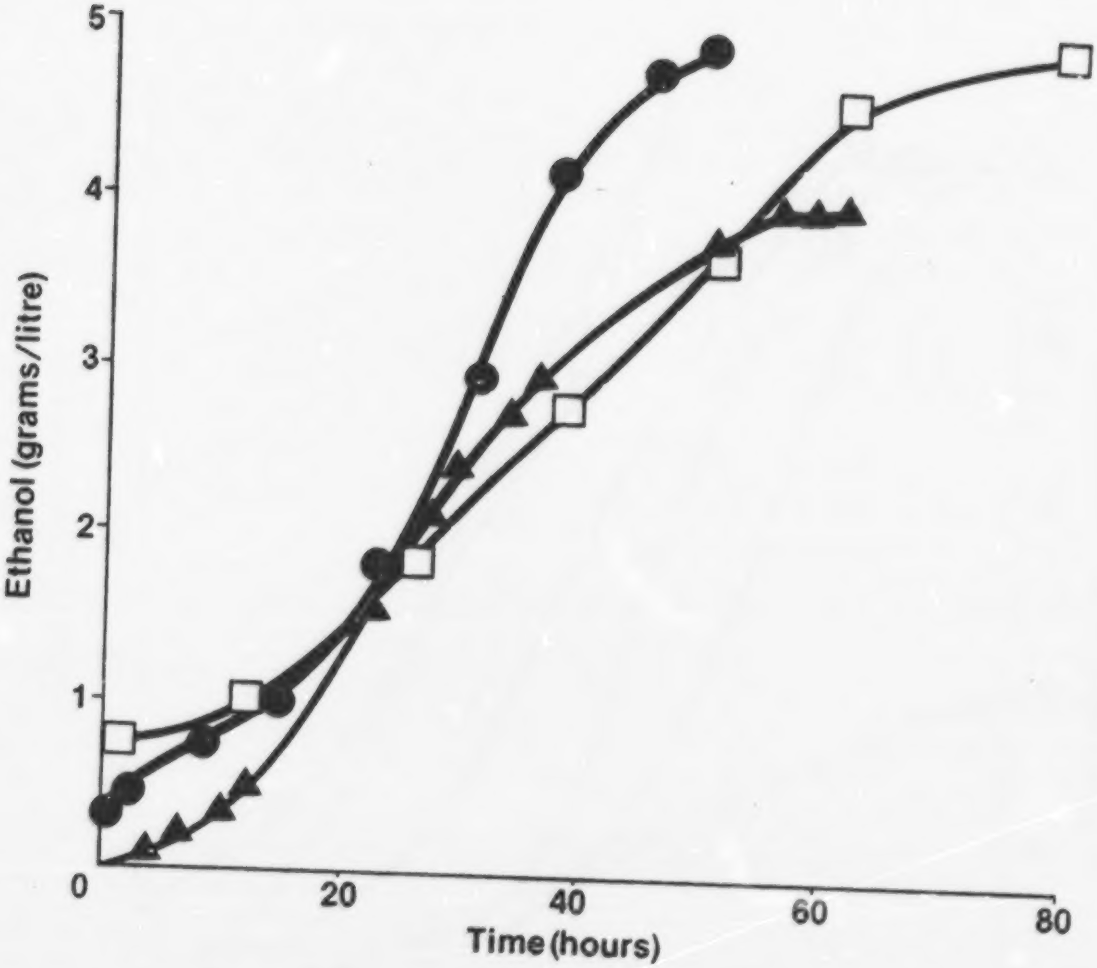


Figure 14. Fermentation of solka floc and corn stover by two strains of *Clostridium thermocellum*. Strain ATCC 27405 solka floc (▲), strain S-7 solka floc (●), strain S-7 corn stover (□). Adapted from Wang *et al.* (434) and Avgerinos *et al.* (439).

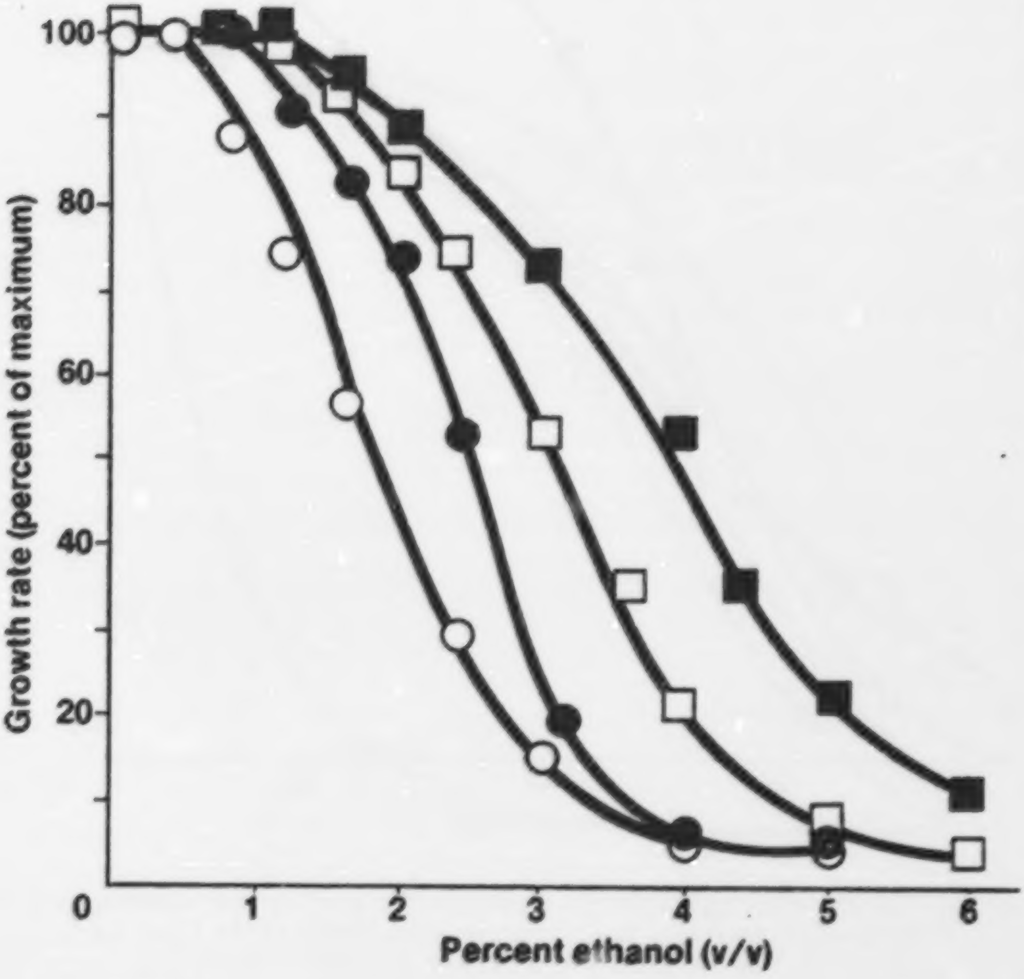


Figure 15. Growth rates of *Clostridium thermosaccharolyticum* isolates in the presence of ethanol. Wild type HG-2 (○), isolate HG-3 (●), isolate HG-4 (□), isolate HG-6 (■).
Adapted from Wang and Fang (693).

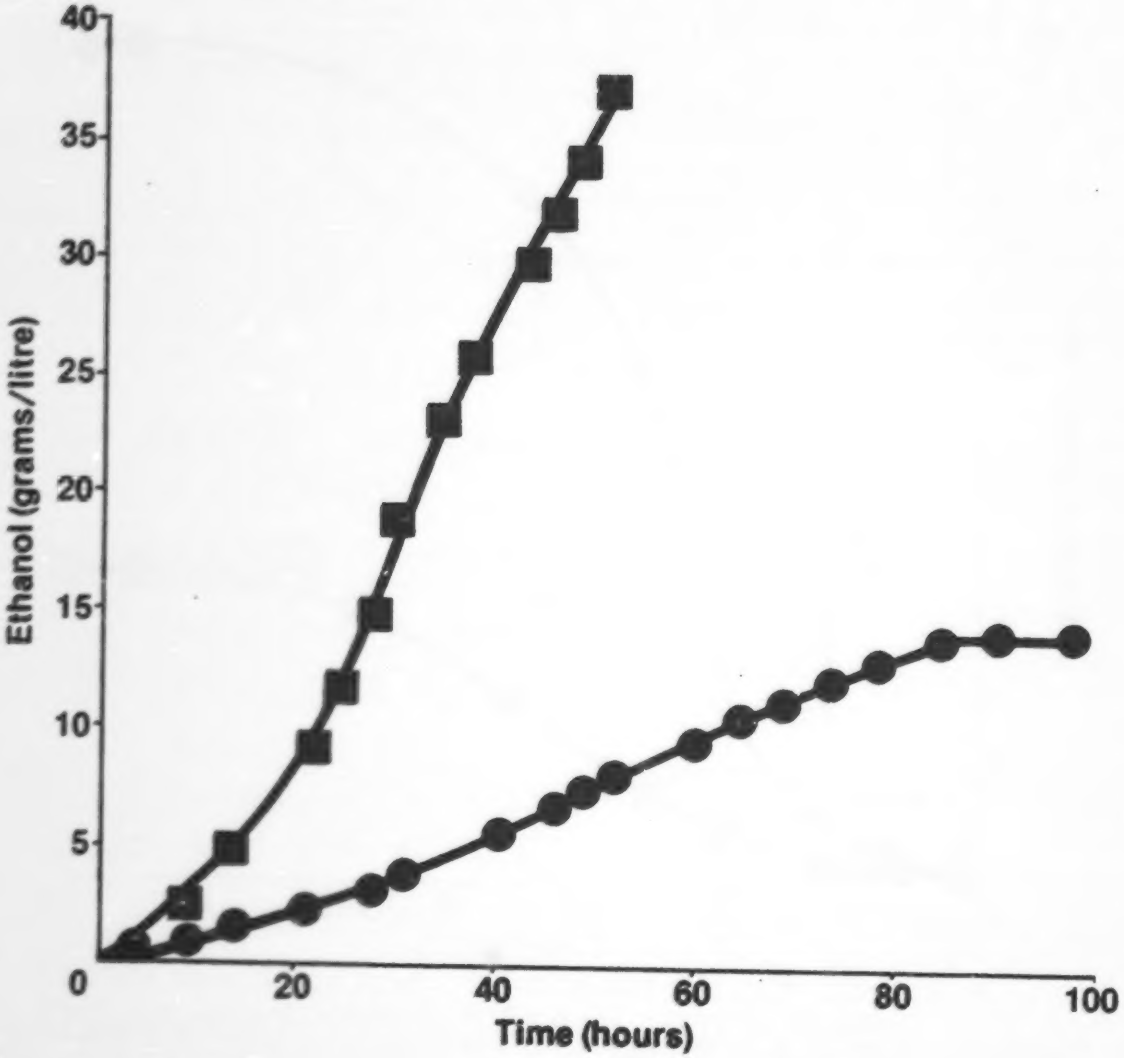


Figure 16. Ethanol production by *Clostridium thermosaccharolyticum* strains HG-2 (●) and HG-6 (■) from xylose.
Adapted from Wang and Fang (693).

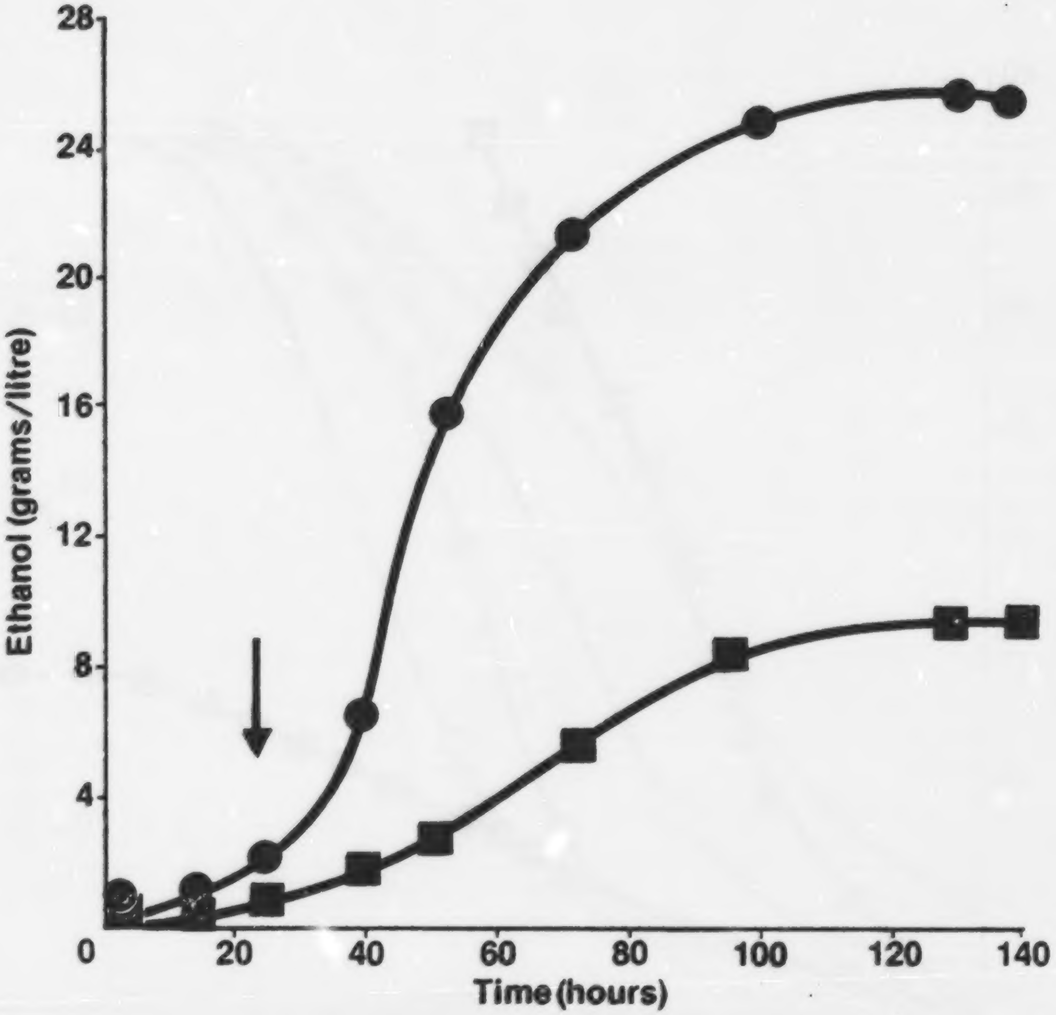
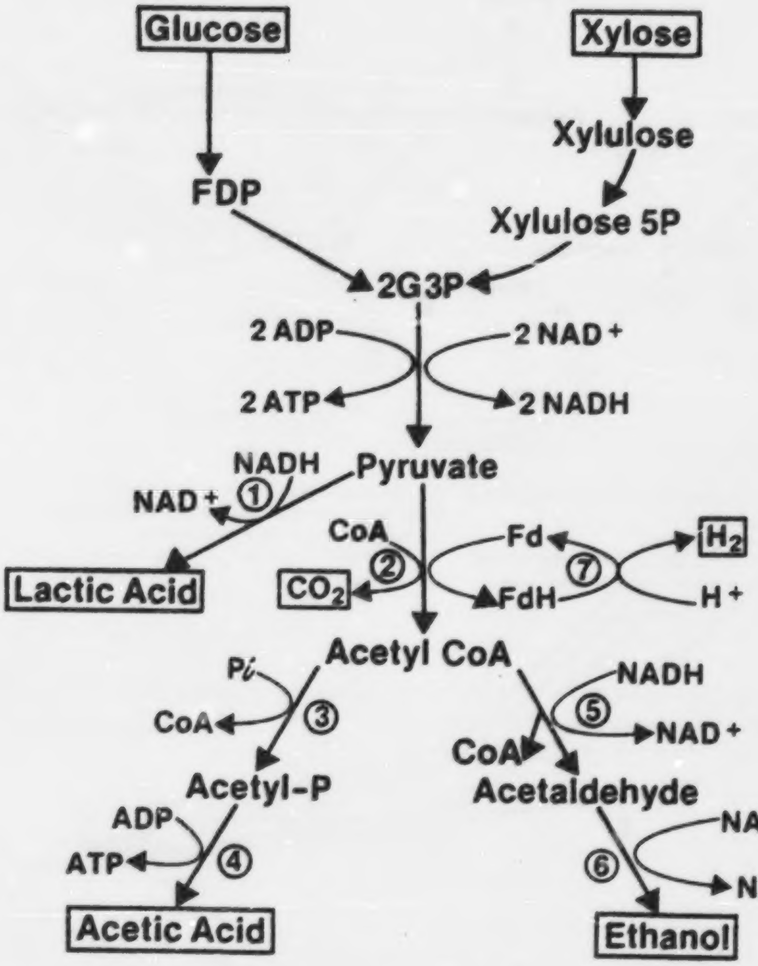


Figure 17. Co-culture fermentation of *Clostridium thermocellum* S-7 and *Clostridium thermosaccharolyticum* HG-4 on solka floc (80 g/ℓ) (●) and corn stover (100 g/ℓ) (■). The time of inoculation of *C. thermosaccharolyticum* HG-4 is indicated by the arrow. Adapted from Avgerinos *et al.* (439) and Wang and Fang (693).



1. Lactate dehydrogenase
2. Pyruvate dehydrogenase
3. Phosphotransacetylase
4. Acetate kinase
5. Acetaldehyde dehydrogenase
6. Alcohol dehydrogenase
7. Hydrogenase and oxidoreductases

Figure 18. Metabolic pathway of typical anaerobic thermophilic ethanologenic bacterium.
Adapted from Zeikus *et al.* (27) and Duong *et al.* (503).

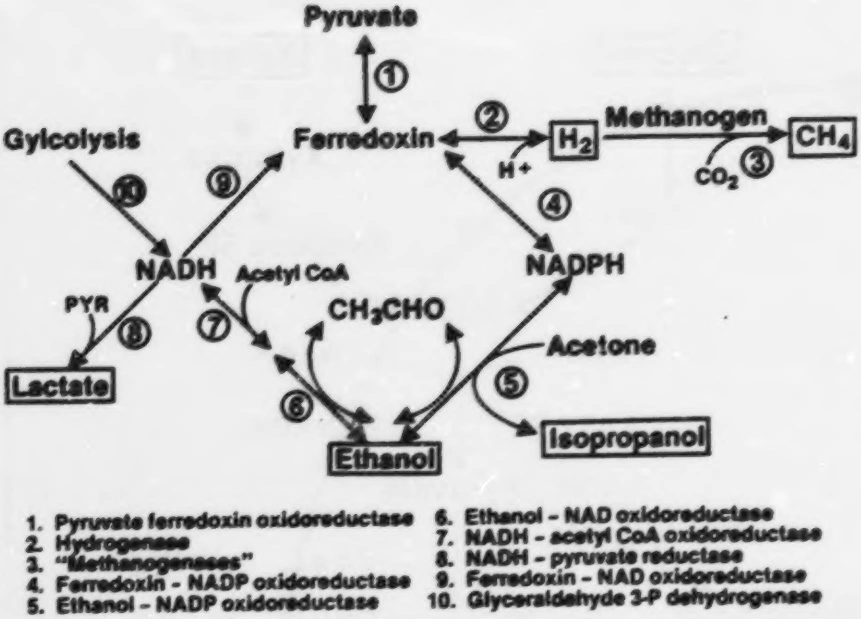

Pyruvate
①
Gylcolysis
Ferredoxin
②
H+
H_2
Methanogen
CH_4
CO_2 ③
⑩
⑨
④
NADH
Acetyl CoA
NADPH
PYR
⑧
⑦
CH_3CHO
Acetone
Lactate
⑥
⑤
Ethanol
Isopropanol
1. Pyruvate ferredoxin oxidoreductase
2. Hydrogenase
3. "Methanogenases"
4. Ferredoxin - NADP oxidoreductase
5. Ethanol - NADP oxidoreductase
6. Ethanol - NAD oxidoreductase
7. NADH - acetyl CoA oxidoreductase
8. NADH - pyruvate reductase
9. Ferredoxin - NAD oxidoreductase
10. Glyceraldehyde 3-P dehydrogenase

Figure 19. Metabolic control of electron flow by reversible oxidoreductases in *Thermoanaerobium brockii*.
Adapted from Ben-Bassat *et al.* (462).

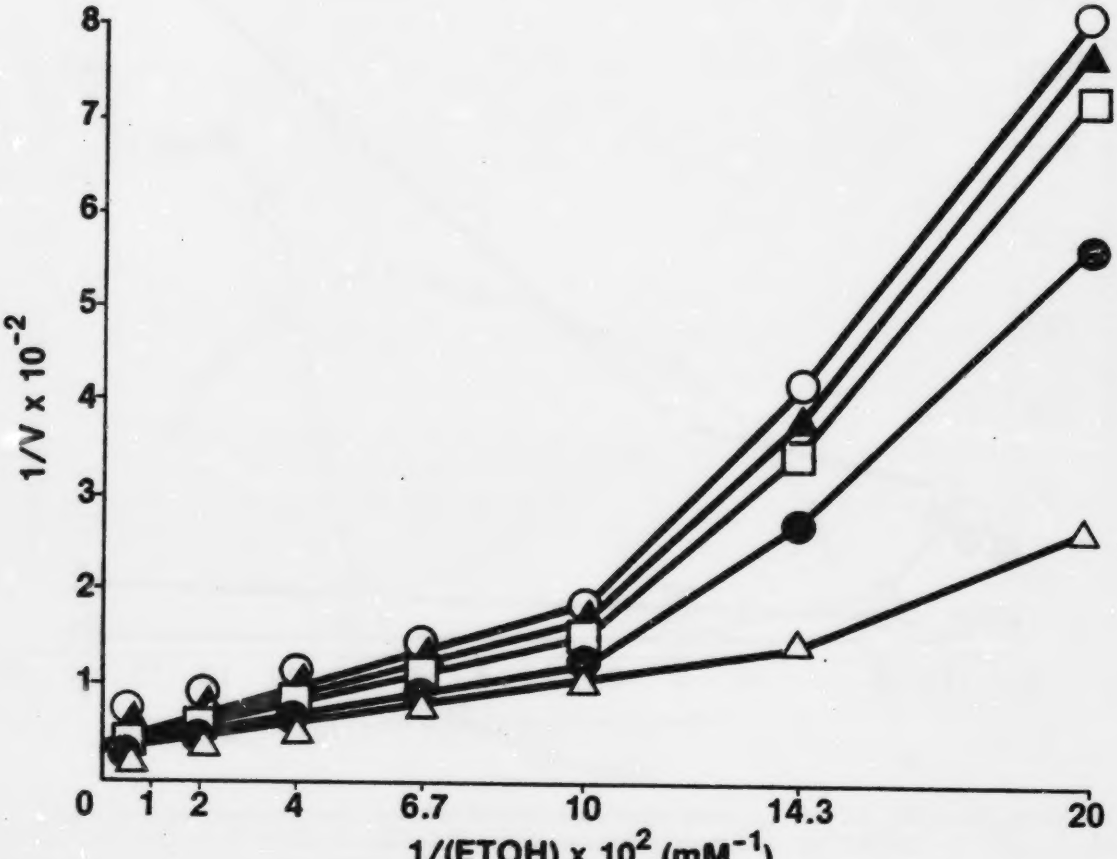


Figure 20. Double reciprocal plots of the initial rates of the alcohol dehydrogenase reaction of *Thermoanaerobacter ethanolicus* using ethanol as the varied substrate and with NADP concentrations of 4 mM (△), 0.5 mM (●), 0.1 mM (□), 0.07 mM (▲), 0.05 mM (○). Adapted from Ljungdahl *et al.* (59).

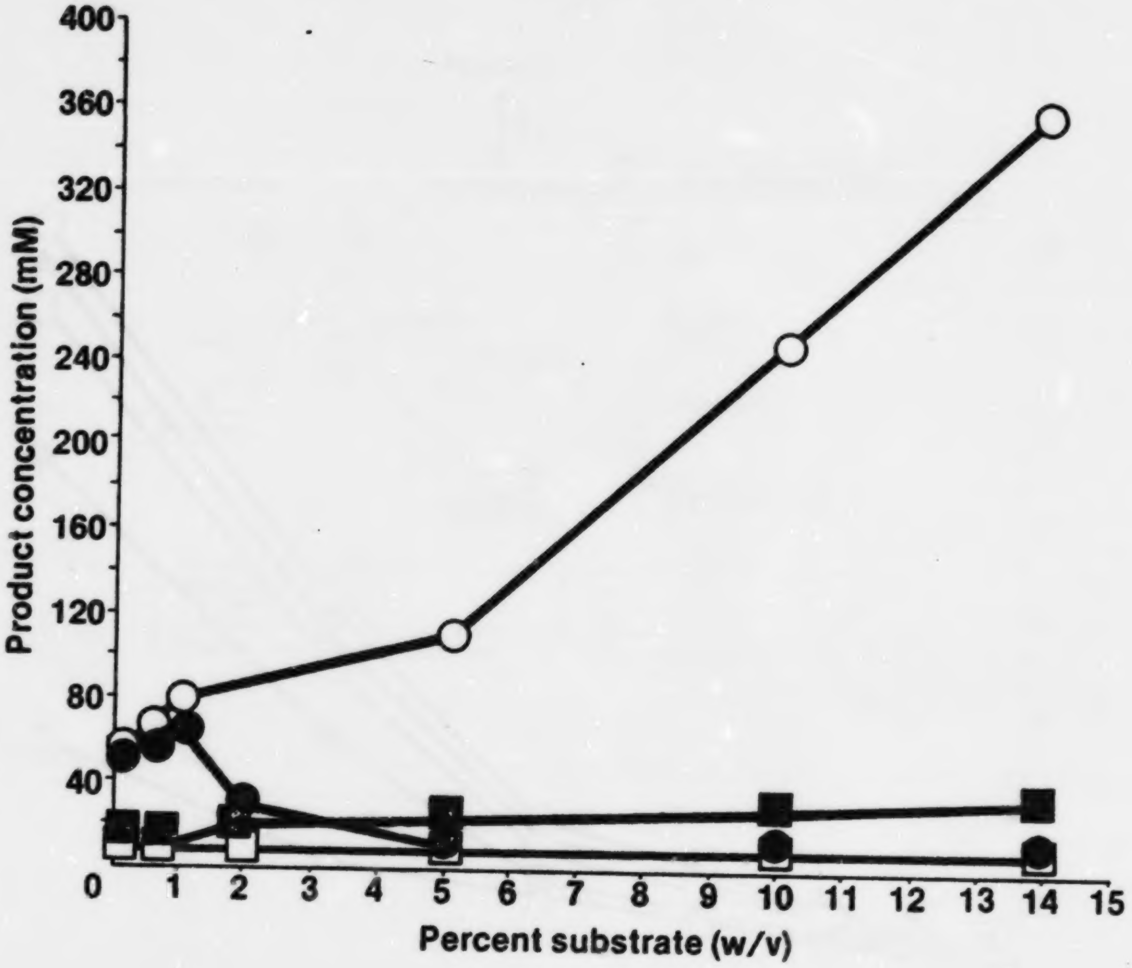


Figure 21. Effect of varying starch concentrations on ethanol and acetate formation by *Thermoanaerobacter ethanolicus* wild type strain JW200 and mutant strain JW200 Fe. Acetate JW200 Fe (□), acetate JW200 (■), ethanol JW200 (●), ethanol JW200 Fe (○). Adapted from Ljungdahl *et al.* (59).

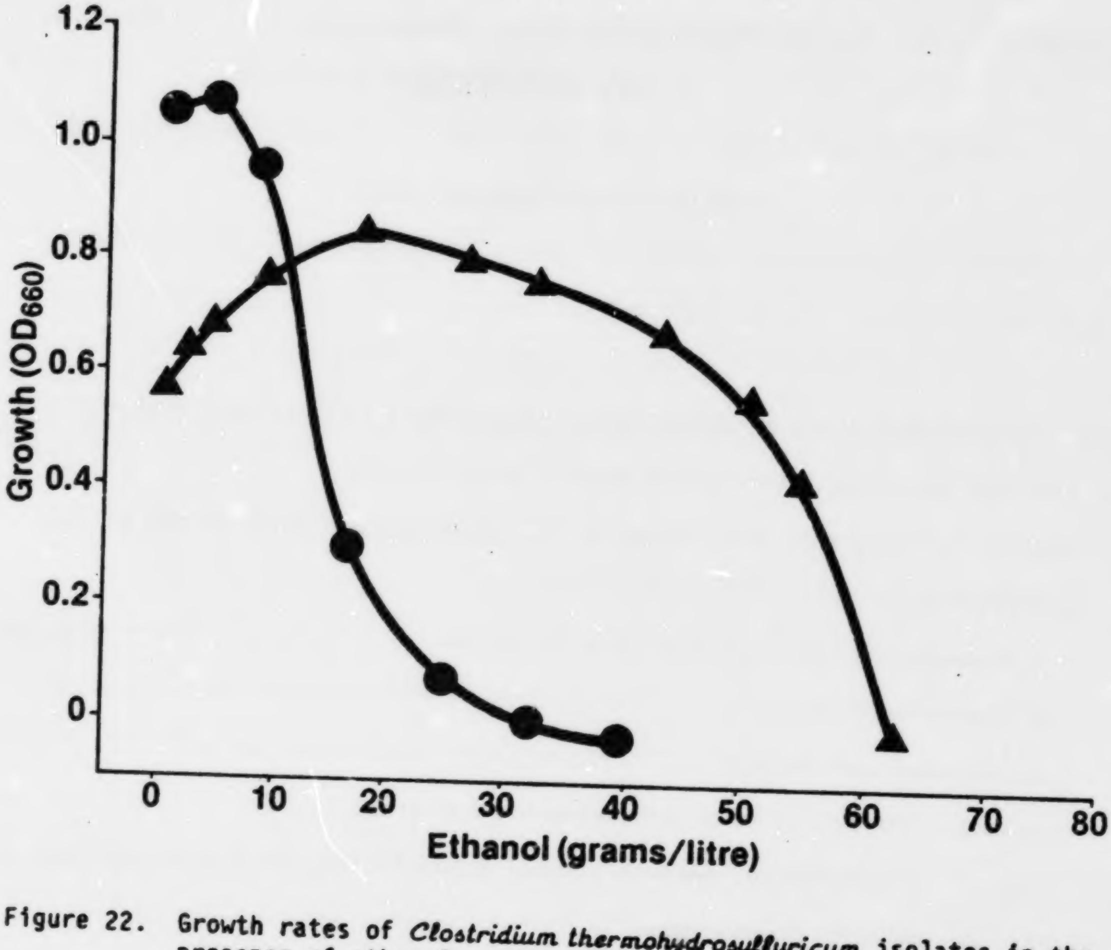


Figure 22. Growth rates of *Clostridium thermohydrosulfuricum* isolates in the presence of ethanol. Wild type 39E (●), mutant 39EA (▲). Adapted from Lovitt *et al.* (492).

XV. APPENDICES

Appendix 1

Recommendations for Developing Economical Processes for Fermentation Ethanol Production at High Temperatures

Recommended Microorganisms

Cellulosics

- *C. thermocellum* or other unidentified thermophilic cellulolytic species (improve cellulase activity and ethanol productivity)
- Examine in co-culture with thermophilic ethanologens (improve ethanol productivity)
  - *T. ethanolicus* - deserves much more attention
  - *C. thermohydrosulfuricum*
  - *C. thermosaccharolyticum*
  - others - search for novel ethanologenic bacteria
    - characterize, measure ethanol productivity, test in co-culture with *C. thermocellum*

(long term)

- Yeasts, *Z. mobilis*, *B. stearothermophilus* - recombinant DNA technology - introduce cellulase genes.

Non-cellulosics

- Yeasts- thermotolerant isolates - examine *Saccharomyces, Kluyveromyces, Candida spp.*
  - improve ethanol tolerance and/or productivity
- *Z. mobilis* - select for growth and fermentation at low pH, improve ethanol tolerance and thermotolerance
- *B. stearothermophilus* - regulatory factors controlling PDH pathway, improve ethanol tolerance
- thermophilic ethanologenic bacteria - as above - especially *T. ethanolicus* (starch, lactose, xylan).

Thermophilic Bacteria

Cellulase Activity (*C. thermocellum*)

Measurements

- standardization for quantitating activity
- test multiple lignocellulosic substrates.

Physiology/Biochemistry

- manipulate and optimize culture conditions (nutrients, agitation, pH, etc.)
  - study regulatory factors
- select deregulated mutants - (hyper-producing strains) - non-metabolizable cellobiose analogs
- improve methods of purification, characterize enzymes, study cellulose/cellulase complex ("yellow substance").

Genetics

- genetic system of *C. thermocellum*, mutation

(long term)

- recombinant DNA - gene banks - clone in genetically characterized mesophilic bacterium (*E. coli*).

Process Design

- large scale, continuous culture, immobilized systems.

Ethanol Yield/Productivity

Physiology/Biochemistry

- manipulate and optimize culture conditions (nutrients, pH, etc.)
- identify nutrient requirements in yeast extract (replace with stillage)
- basis of ethanol tolerance - intracellular ethanol, target sites
- examine sensitivities to inhibitors in lignocellulosics
- study regulatory factors - intra- and inter-specific metabolic control
  - carbon and electron flow to other products
    - ferredoxin-NAD(P) oxidoreductases, hydrogenases - H_2, exogenous electron acceptors
    - control of PDH pathway (*B. stearothermophilus*)
    - regulation of enzymes
      - FDP-dependent LDH - intracellular FDP
      - NAD(P)-dependent ADH - ethanol inhibition
    - regulation of morphology - *C. thermosaccharolyticum* - elongated, non-dividing cells - explore novel continuous dilution culture system.

Genetics

- select for mutants - eg. ADH-deregulatory, LDH negative mutants
- adaptation, mutation, selection for ethanol tolerant isolates - (select on mixed carbohydrates)

(long term)

- recombinant DNA - purification and characterization enzymes and plasmids - gene banks
- clone thermophilic genes into mesophilic bacteria with well defined genetic systems - recover thermostable products by thermal separation
- clone gene for ADH to increase copy number
- study mesophilic transformant - determine genes responsible for heat stability - map genes.

Process Design

- design fermentation processes that maximize H_2 in gas phase for *C. thermocellum* cultures
- examine performance in large scale bioreactors - strain stability, heat stress on equipment (steel fermentors, sensors), effects of high substrate concentrations, evaporation problems, actual cooling and heating requirements, O_2 requirements (aerobic organisms), actual distillation costs
- examine continuous systems of ethanol recovery
  - CO_2 stripping, vacuferm - performance, economics, long term effects - research for novel systems of on-line extraction.

Yeasts

Screening/Selection

- screen culture collection at high cell density for thermotolerant isolates - especially examine ethanol tolerant strains (wild type, mutants, fusion products, etc.)
- examine growth and/or fementation ability (plates, ethanol values, Durham tubes, temperature gradient incubator)
- examine survivors of heating process
- adaptation - exposure to progressively higher temperatures and/or ethanol concentrations

(long term)

- chemostat, novel continuous selection feedback control system - based on CO_2 production, research new selection strategies.

Physiology/Biochemistry

- conduct extensive study on one strain of *S. cerevisiae* of defined genetic background - examine effect of physiological factors on growth and/or fermentation at high temperature (fatty acids, sterols, minerals, sugars, oxygen, etc.)
- basis of thermotolerance - thermostability of cellular components - proteins (type of bonding, glycosylation), membranes (lipid composition, phase transitions), membrane/protein interactions, nucleic acids (T_m, unique bases) - interaction with ribosomal proteins
- determine if yeasts can regulate their internal temperature and if water loss is accentuated at high temperatures

- role of trehalose and polyhydroxy compounds (polyols, carbohydrates and their derivatives) - response to generalized stresses, thermotolerance
- heat shock - examine heat-shocked yeasts for ethanol tolerance and/or fermentation ability, determine if hsp's necessary for acquisition of thermotolerance, determine other stimuli that induce hsp's or heat shock gene expression (minerals, trauma, recovery from anoxia, etc.) - effects of these factors on thermotolerance in yeast should then be assessed.
- basis of ethanol tolerance - intrinsic and extrinsic factors - intracellular ethanol - target sites (uncoupler, inhibitor of enzymes, and transport), effect of ethanol on membranes and temperature profiles
- study regulatory factors - eg., fatty acid synthesis, trehalose.

Genetics

- mutation selection - ethanol tolerance and/or thermotolerance
- classical genetics - mating, spheroplast fusion

(long term)

- recombinant DNA - gene banks - clone structural or regulatory genes for - fatty acid synthesis or unique lipids (*L. heterohiochii*) or hsp's.

Process Design

- examine performance in large scale bioreactors - especially with high cell density - recycling, immobilized systems, continuous culture
- examine potential in SSF cellulose processes
- determine if on-line extraction of ethanol increases productivity - if so, then test novel systems of ethanol recovery - long term effects, economics (CO_2 stripping, vacuferm, develop new methods).

Appendix 2

The following is a list of people who have provided helpful discussions and/or personal communication of results prior to publication:

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Dr. B.F. Johnson	National Research Council, Canada
Dr. A.W. Khan	National Research Council, Canada
Dr. G. Mahmourides	National Research Council, Canada
E. Meek	National Research Council, Canada
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